1 **Removal characteristics of DON in pharmaceutical wastewater and its**
2 **influence on the *N*-nitrosodimethylamine formation potential and acute**
3 **toxicity of DOM**

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Abstract

Previous research has focused on dissolved organic carbon (DOC) as a surrogate for dissolved organic matter (DOM) in pharmaceutical wastewater. Dissolved organic nitrogen (DON) as a part of DOM has received little attention. This study investigated the removal characteristics of DON and its influence on the *N*-nitrosodimethylamine formation potential (NDMA FP) and acute toxicity of DOM in a full-scale hydrolysis/acidification + anaerobic/anoxic/aerobic + moving bed biofilm reactor (MBBR) process treating pharmaceutical wastewater. Results showed that maximum removal of DON ($68 \pm 12\%$) was present in the anaerobic process. The removal of DON by anoxic and aerobic processes was negligible as a result of the production of new N-containing compounds that are characteristic of proteins/amino sugars and lipids. DON concentration decreased significantly in the MBBR process ($p < 0.05$, *t*-test), indicating that manipulation of the solids retention times (SRTs) could be a solution to minimize DON. Based on the Pearson correlation analysis, the behavior of NDMA FP and DOM acute toxicity was significantly associated with the 3 kDa<MW<10 kDa ($r = 0.709$, $p < 0.05$) and MW<3 kDa DON ($r = 0.659$, $p < 0.05$), respectively, and are not identical to that of DOC fractions ($r = 0.037$ – 0.466 , $p = 0.051$ – 0.886). Moreover, the removal and molecular changes of DON are not coupled with that of DOC during biotreatment. Thus, testing the performance indicator of DON in pharmaceutical wastewater was recommended, as it provides important information for DOM removal characteristics.

Keywords: Dissolved organic nitrogen (DON), Dissolved organic matter (DOM), Pharmaceutical wastewater, Biotreatment, *N*-nitrosodimethylamine (NDMA), Acute toxicity

1. Introduction

Pharmaceutical industries consume large amounts of water in the pharmaceutical manufacturing processes and generate highly polluted toxic wastewaters (Gadipelly et al., 2014). Biological treatment, which includes anaerobic and aerobic treatment systems, has been traditionally employed by industry for dealing with pharmaceutical wastewater (Chen et al., 2011b; Gadipelly et al., 2014). Dissolved organic matter (DOM) is the main source of contaminants in the biotreatment system, and knowledge of its behavior can help improve and optimize treatment performance (Yekta et al., 2012). DOM is a heterogeneous mixture of various dissolved organic carbon (DOC) and nitrogen (DON) forms (Michael-Kordatou et al., 2015). DON analysis is more analytically challenging than DOC (Lee and Westerhoff, 2005; Xu et al., 2010). Previous research, therefore, has focused on DOC or total organic carbon (TOC) as a surrogate for DOM in pharmaceutical wastewater (Melero et al., 2009; Sirtori et al., 2009; Mascolo et al., 2010; Lester et al., 2013). Little is known about the behavior of DON in pharmaceutical wastewater, although the pharmaceutically organic compounds contain DON (Sirtori et al., 2009; Lester et al., 2013).

Recently, DON as a part of DOM in wastewater has become a concern due to its impacts on the potential formation of nitrogenous disinfection by-products (N-DBPs)

with higher carcinogenicity compared with carbonaceous DBPs (C-DBPs) (Lee et al., 2007). *N*-nitrosodimethylamine (NDMA) is an important N-DBP preferentially formed by chloramines (Yoon et al., 2013). Some pharmaceutically nitrogenous organic compounds have been found to be potent precursors for NDMA (Roux et al., 2011). The study proposed by Yoon et al. (2011) also showed the formation of significant amounts of NDMA (up to 7020 ng L⁻¹) in influent wastewater of a municipal wastewater treatment plant (WWTP), which receives industrial wastewater from pharmaceutical factories. This presents a risk that harmful N-DBPs and their precursors originating from industrial sources enter the drinking water supply if wastewater-impaired water resources are used (Krauss et al., 2009). Thus, the DOM or DON associated with NDMA precursors in pharmaceutical wastewater should be minimized by the WWTP before being discharged into receiving waters. Identifying the DOM or DON responsible for the NDMA precursors in pharmaceutical wastewater is the first step toward that goal.

DOM compounds in pharmaceutical wastewater are associated with toxic risk (Ji et al., 2013a). A previous study (Chen et al., 1999) used the luminescent bacterium, *Photobacterium phosphoreum*, as the test microorganism to assess the toxicity of the discharges in the highly intensive pollution industries and observed a positive relationship between the organic loads and toxic discharges. In contrast, Köhler et al. (2006) reported an increase in biotoxicity with substantial TOC reduction in biological industrial wastewater treatment. A more recent research conducted by Ji et

al. (2013b) found that some nitrogenous organic compounds in pharmaceutical wastewater are associated with toxic risk, indicating that pharmaceutical DON is related to the toxicity as well. However, to our knowledge, the influence of DON on the biotoxicity of DOM in pharmaceutical wastewater is rarely investigated. Bioluminescence inhibition assay of luminescent bacteria is widely used for the acute toxicity assessment of pollutants and has also been carried out in the field of pharmaceutical wastewater (Ji et al., 2013a,b). So, the luminescent bacterium assay was carried out to determine the acute toxicity of DOM in pharmaceutical wastewater.

Wastewater-derived DON is extremely complex. Pehlivanoglu-Mantas and Sedlak (2008) measured the specific organic nitrogen compounds in wastewater; however, almost 70% of the DON was unidentifiable. The complexity of DON can be characterized in terms of molecular weight distribution by ultrafiltration membranes and hydrophobicity-based fractionation by XAD resin fractionation (Lee et al., 2007; Dwyer et al., 2009; Liu et al., 2012). Different DON fractions differ greatly in chemical composition and characterization (Lee et al., 2007; Hu et al., 2016). Therefore, measurement of DON fractions can enable a better understanding of the relationship between DON and NDMA precursors and the biotoxicity of DOM. In recent years, electrospray ionization coupled to Fourier transform ion cyclotron resonance ultrahigh resolution mass spectrometry (ESI-FTICR-MS) has been used to determine the molecular composition of wastewater-derived DON (Mesfioui et al., 2014). ESI-FTICR-MS allows for an unprecedented resolution of thousands of

molecular formulas from organic matter mixture, which provides detailed molecular transformations information on DON (Yekta et al., 2012; Lusk and Toor, 2016).

Accordingly, the first objective of this study was to characterize the removal of DON in a full-scale biological WWTP, which treats pharmaceutical wastewater. High resolution mass spectrometry ESI-FTICR-MS was applied to provide a better understanding of the removal characteristics of DON at the molecular level. The second objective was to investigate the influence of DON on the NDMA formation potential (FP) and acute toxicity of DOM. Correlations of the DON fractions to the NDMA FP and acute toxicity were assessed based on Pearson correlation analysis. For the first time, this study provides an overview of how the DON in pharmaceutical wastewater changes along the biotreatment traits, which is beneficial to understand the roles of WWTP biotreatment units in the removal characteristics of the DOM.

2. Materials and methods

2.1. Description of the full-scale WWTP and sample collection

Samples were obtained from a full-scale hydrolysis/acidification (HA) + anaerobic/anoxic/aerobic (A1/A2/O) + moving bed biofilm reactor (MBBR) WWTP treating pharmaceutical fermentation industry wastewater, which came from a large-scale drug and fine chemical manufacturing industrial plant located in Taizhou, China. This factory is focused on the production of specific drugs for cancer treatment. The effective reactor volumes of the HA, A1, A2, O, and MBBR reactors were 4,000 m³, 6,000 m³, 2,400 m³, 3,000 m³, and 3,000 m³, respectively. The WWTP has a peak

pumping capacity of 2,400 m³ day⁻¹ and an average flow of 2,000 m³ day⁻¹. The hydraulic retention time (HRT) was 2 d, 3 d, 0.6 d, 0.75 d, and 0.75 d for HA, A1, A2, O, and MBBR reactors, respectively. The solids retention time (SRT) was 62 d, 41 d, and 34 d for A1, A2, and O reactors, respectively. The sampling was performed at six key locations within the full-scale biotreatment plant (Fig. 1). Wastewater obtained from each location was immediately encapsulated in a 4 °C container and conveyed to a lab in 24 h. Three sampling campaigns were carried out in Apr–2015, Jul–2015 and Jan–2016. Each month, three wastewater samples (5 L per sample) were collected on Day 1, Day 15, and Day 29. They were stored at 4 °C and were combined prior to laboratory analysis. Characterization of the pharmaceutical wastewater in different locations are included in Table S1 in the Supporting Information (SI). All samples were analyzed for DON, DOC, NDMA FP, and acute toxicity. During one sampling event (Jan–2016), samples were also collected for ESI-FTICR-MS analyses.

2.2. DON and DOC fractionation

Supelite DAX–8 resin (Sigma-Aldrich, USA) was used to divide DON and DOC into hydrophobic (HPO) and hydrophilic (HPI) fractions. Molecular weight (MW) fractionations were conducted in a 400 mL commercial stirred cell unit (Model 8400, Millipore Corp. USA), employing filters with MW cutoffs of 30, 10, and 3 kDa (Millipore Corp. USA). Details of the resin and size separation protocol can be found in Hu et al. (2016).

2.3. DOM extraction method

100 mL of each of the diluted samples (COD: $\sim 250 \text{ mg L}^{-1}$) was acidified with hydrochloric acid to pH 2 and pumped through the SPE cartridge (functionalized styrene-divinyl-benzene polymer resin (PPL), 1g, Supelco, USA). This resin has been proposed for DOM isolation from wastewater (Yekta et al., 2012), which is more efficient and reproducible than sorbents of a silica structure bonded with different hydrocarbon chains (Varian C8, C18, C18OH, and C18EWP) (Dittmar et al., 2008). After the solution had passed through the resin, the cartridge was rinsed with at least two cartridge volumes of acidified LC-MS water (Merck Lichrosolv, Germany) for complete removal of salts. DOM sorbents were dried under vacuum and then eluted with 10 mL of LC-MS methanol (Merck Lichrosolv, Germany). On the one hand, eluted DOM samples were diluted (dilution factor of 10) and run in 100% LC-MS methanol for ESI-FTICR-MS analysis. On the other hand, eluted DOM was completely dried under a nitrogen flow. Next, the dry residues were dissolved in 3% NaCl distilled water for the DOM acute toxicity measurement. Scheme of the DOM extraction and their uses in the following tests are included in Fig. S1 in the SI.

2.4. NDMA FP tests

Since the concentration of DON and DOC in wastewater samples were high (Table 1), excess of disinfectant cannot be guaranteed in a 1L batch reactor. Therefore, filtered wastewater samples were diluted (diluted concentrations: $150\text{--}300 \text{ mg COD L}^{-1}$) and then conducted in batch reactors with freshly prepared monochloramine stock solution (final concentration of 2.0 mM monochloramine (Yoon et al., 2011)) and incubated in

the dark at pH 6.9 and 25 °C. The residual monochloramine concentrations at the end of the test were greater than or equal to 0.09 mM, implying that the chloramines concentration was sufficient for NDMA formation throughout the chloramination. After 10 days of contact time, NDMA was extracted using an SPE cartridge (Coconut charcoal, 2g, Anpel, China) and was then measured by LC-MS/MS (Xevo-TQ, Waters, USA). The method detection limits (MDLs) was 2 ng L⁻¹. Average recovery and relative standard deviations (RSDs) were 106 ± 7% and 99 ± 5% in Milli-Q water and wastewater, respectively. NDMA and NDMA-d₆ were obtained from Supelco Inc. (USA). Milli-Q water, obtained from a Millipore water system (Millipore, Bedford, MA, USA), was used to prepare all the required reagents.

2.5. DOM acute toxicity measurement

Acute toxicity of DOM was expressed in the luminescence inhibition rate of luminescent bacteria (*Photobacterium phosphoreum* T3 mutation, obtained from the Nanjing Institute of Soil Science, Chinese Academy of Sciences) (Wu et al., 2016). After 10 min of exposure, the acute toxicity was determined by using the Microtox Toxicity Analyzer (DXY-3, made by the Nanjing Institute of Soil Science, Chinese Academy of Science). The acute toxic unit (TU_a) was chosen as a way to estimate the toxicity of DOM: $TU_a = 100/EC_{50}$, where concentrations causing 50% inhibition of luminescence (EC₅₀) were obtained by regression analyses of the diluted concentrations and the luminescence inhibition rates (Wu et al., 2016). Each sample was tested in quintuplicate.

2.6. Ultrahigh-resolution mass spectrometry analysis

SPE-extracted DOM was analyzed using a Bruker SOLARIX 7 T FTICR-MS in combination with a negative mode ESI. A sample flow rate was maintained at 120 mL/h and the ESI voltages for each sample were maintained at 3.4 kV on the spray shield and 4.1 kV on the capillary under 200 °C. A methanol blank was injected before and after each sample to ensure no cross-contamination between samples. For each sample, 300 scans were acquired. Mass peak lists were based on a signal-to-noise ratio of 5 and masses within the mass range of 200–600 Da were considered. All mass spectra were calibrated following Chen et al. (2014), and molecular formula calculations were included on the following atoms: $^1\text{H}_{2-120}$, $^{12}\text{C}_{2-50}$, $^{16}\text{O}_{0-30}$, $^{14}\text{N}_{0-6}$, $^{32}\text{S}_{0-2}$, and $^{31}\text{P}_{0-2}$.

2.7. Chemical analysis

The concentrations of DON were calculated as the difference between total dissolved nitrogen (TDN) and the sum of dissolved inorganic nitrogen (DIN) including ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) (Lee and Westerhoff, 2005; Liu et al., 2012). A dialysis-based pretreatment technique was adopted to reduce the interference from DIN to the quantification of DON (Lee and Westerhoff, 2005). Ammonium (NH_4^+) was determined by the salicylate method (EPA of China, 2002). Nitrate (NO_3^-), nitrite (NO_2^-) and total dissolved nitrogen (TDN) were analyzed by ion chromatography (Dionex ICS-1100, USA). Dissolved organic carbon (DOC) was measured using a Shimadzu 5000-A TOC analyzer (Shimadzu, Japan). The

concentration of monochloramine was measured by *N,N*-diethyl-*p*-phenylenediamine colorimetry (EPA of China, 2002).

2.8. Statistical analyses

SPSS 19.0 (IBM, Armonk, New York) was used for *t*-test, one-way ANOVA, and Pearson correlation analysis. Two-dimensional (2D) correlation was performed using the “2D Shige” software released by Kwansei-Gakuin University, Japan.

3. Results and discussion

3.1. Removal characteristics of the DON along the biotreatment traits

3.1.1 Removal of DON

The DON concentration in wastewater samples cannot be quantified directly, and two most commonly-used DON analysis methods involve: (1) subtracting NH_4^+ from total Kjeldahl nitrogen (TKN) and (2) subtracting dissolved inorganic nitrogen (DIN) (i.e., NH_4^+ , NO_2^- , and NO_3^-) from total dissolved nitrogen (TDN). TKN analysis may be interfered by the presence of high concentrations of NO_3^- ($\geq 10 \text{ mg L}^{-1}$) (Pehlivanoglu-Mantas and Sedlak, 2006), making it unreliable for the samples of this study (NO_3^- concentrations ranged from 38 to 288 mg L^{-1}). For the second method, the accuracy of the DON measurement relied strongly on the ratio of DIN to TDN (Lee and Westerhoff, 2005). Due to the high DIN/TDN ratios (> 0.8 , Fig. S2 in the SI) in the pharmaceutical wastewaters, a dialysis pretreatment was used to remove DIN prior to determination of DON (Lee and Westerhoff, 2005).

DON averaged 61.49–70.58 mg L^{-1} in the primary effluent (S1) and 10.20–14.11

234 mg L⁻¹ in the MBBR effluent (S6) corresponding to 77–84% removal of DON by the
235 biotreatment (Table 1). The majority of DON was removed in the A1 process (68 ±
236 12%, Fig. 2), which agrees with Huo et al. (2013) reporting that the major removal of
237 DON occurred during the anaerobic process in a biological nutrient removal
238 municipal wastewater treatment system. The concentration of DON stayed almost
239 constant during the A2 process (Table 1, Fig. 2). A slight increase in DON (2.47–4.27
240 mg L⁻¹) was observed after the O process except on the second sampling event (Table
241 1). The possible reason for the increase in DON is the produced soluble microbial
242 products (SMPs) during the biological process. This hypothesis will be further
243 confirmed in Section 3.1.2. DON concentrations in MBBR effluent (S6) (12.07 ± 1.96
244 mg L⁻¹) were significantly lower than that in the O effluent (S5) (23.41 ± 3.93 mg L⁻¹,
245 $p = 0.035$, t -test). The process of MBBR with the addition of freely moving plastic
246 carriers has the advantage of greater solids retention times (SRTs) compared to
247 conventional activated sludge process (Shi et al., 2015). In this way, slow-growing
248 organisms can be kept in the system without being washed out. MBBR is operated
249 similarly to the O process in this study, except that biofilm grows on small carriers. It is,
250 therefore, indicated that manipulation of the SRTs could be used as a method for
251 minimizing the DON in pharmaceutical wastewater. In fact, findings about longer
252 SRTs contributing to less amounts of DON have been reported in municipal biological
253 nutrient removal (BNR) WWTPs (Sattayatewa et al., 2010). Interestingly, recent
254 research conducted by Simsek et al. (2016) indicated no trend between effluent DON

and SRTs in a laboratory-scale activated sludge system. This difference may be due to the better adaptation of the slow-growing microorganisms in the BNR WWTPs since SRTs applied in the BNR process (8.38–25.3 days, Sattayatewa et al., 2010) were higher than that of the laboratory-scale activated sludge system (0.3–13 days, Simsek et al., 2016).

A decreasing trend in DOC concentration along the biotreatment train was seen in the first two units and remained stable in the last three units (Table 1, Fig. 2). Maximum removal of DOC (66–69%) was present in the HA process. HA has been a widely used bioprocess prior to the anaerobic system, which could not only improve the biodegradability but also reduce the chemical oxygen demand (COD) (Yang et al., 2015; Wu et al., 2016). In contrast, only 6–18% of DON was removed in HA (Fig. 2). Different removal characteristics between DON and DOC resulted in the changes of the DON/DOC ratio, ranging from 0.031 to 0.194, along the biotreatment trains (Table 1). Statistical analyses showed no significant difference in DON and DOC concentrations in all the locations among the three sampling events ($p > 0.05$, one-way ANOVA test, Table 1). Hence, biological process had a stronger effect on the variation of DON and DOC than any temporal changes of the environmental conditions (e.g., temperature).

3.1.2 Molecular compositional pattern of DON

High resolution mass spectrometry ESI-FTICR-MS was applied to provide a better understanding of the removal characteristics of DON along the biotreatment traits at a

molecular level. To extend the observations, entire N-containing formulas were analyzed with van Krevelen diagrams, which were constructed using the molar ratio of hydrogen to carbon (H/C ratio) as the ordinate and the molar oxygen-to-carbon ratio (O/C ratio) as the abscissa (Kim et al., 2003). Major biogeochemical classes of compounds have their own characteristic H/C or O/C ratios. As a result, each class of compounds plots in a specific location on the diagram (Antony et al., 2014). These compounds include lignin, condensed aromatics, unsaturated hydrocarbons, tannins, proteins/amino sugars, lipids, and carbohydrates. The number of peaks of N-containing formulas varied along the biotreatment trains, which were 352, 389, 500, 309, 311, and 419 of the primary effluent (S1), HA effluent (S2), A1 effluent (S3), A2 effluent (S4), O effluent (S5), and MBBR effluent (S6), respectively (Fig. S3 in the SI). The change trend of peaks number along the biotreatment units was inconsistent with the results of the DON (Fig. S3 in the SI). This is because the ESI-FTICR-MS is not a quantitative technique and the observed point on the van Krevelen diagram is based on numbers of formulas (Mesfioui et al., 2014).

However, this technique is capable of distinguishing between new production and degradative loss to evaluate the extent of DON transformations (Lusk and Toor, 2016). The number of peaks of N-containing formulas highly increased after the A1 process indicating that while some N-containing components were removed, some new chemical compounds were produced. Production of new compounds was most likely a result of the produced SMPs and release of N-containing compounds as a result of

degradation of SMPs during the biological process. SMPs, which are released by microorganisms during normal metabolism in biological processes, are composed of proteins, nucleic acids, polysaccharides, and some humic-like materials (Xie et al., 2016). Except for polysaccharides, these biologically derived components are nitrogen-containing organic compounds. SMPs, therefore, represent a significant fraction of the DON (Sattayatewa et al. 2009). The percentage of N-containing molecules at proteins/amino sugars plus lipid region from HA effluent (S2) (42%), A1 effluent (S3) (43%), A2 effluent (S4) (45%), O effluent (S5) (44%), and MBBR effluent (S6) (42%) were larger than that from primary effluent (S1) (37%, Fig. S4 in the SI). The high presence of N-containing formulas that are characteristic of amino sugars, proteins, and lipids in samples from S2-S6 confirmed these as biologically derived components during the biological process (Antony et al., 2014). Specially, the highest percentage of N-containing molecules in the proteins/amino sugars plus lipid region occurred in A2 effluent (S4) (45%) and O effluent (S5) (44%, Fig. S4 in the SI). It is not surprising then, the low removal efficiency of DON occurred in the A2 and O process, considering the higher abundance of biologically derived materials.

2D correlation analysis of H/C ratios of the CHON and CHO formulas results in a 2D map that highlights the major changes in the H/C ratios of formulas for samples along the biotreatment traits (Fig. 4). For CHON formulas, the correlation peaks intensity is greatest at H/C ratios of ca. 1.25–1.62, indicating that most of the changes occur in these regions during biotreatment (Mesfioui et al., 2014). The white space

implies that these CHON formulas do not display significant changes in number of formulas during biotreatment. A different change was observed when the 2D correlation was applied to the CHO formulas. As shown in Fig. 4b, one positive (H/C ca. 1.50–1.88) and one negative (H/C ca. 1.13–1.38) off-diagonal cross correlation were observed. The different cross correlation peaks of CHON and CHO indicates that the changes of DON and DOC formulas in pharmaceutical wastewater during biotreatment are not coupled.

Overall, both the removal and molecular changes of DON were different from that of DOC (Table 1, Fig. 4). The concentration of biological effluent DON in this study ($12.07 \pm 1.96 \text{ mg L}^{-1}$) was considerably higher than that from municipal wastewater ($1.18 \pm 0.33 \text{ mg L}^{-1}$, see Bratby et al. (2008) for a review). Moreover, the occurrence of a large number of lignin-like N-containing formulas from MBBR effluent (S6) (36%, Fig. S4 in the SI) reflects the potential aromatic nature of these compounds which likely limits their degradation during biological processes (Yekta et al., 2012). Notice that DOM includes both DOC and DON (Michael-Kordatou et al., 2015). Therefore, mere reduction of the DOC is not sufficient for ensuring an environmental impact reduction of DOM in pharmaceutical wastewater, taking the harm of DON on environment into consideration.

3.2. Correlation of DON fractions and NDMA FP in pharmaceutical wastewater

Correlations of the DON and DOC fractions to the NDMA FP were assessed based on Pearson correlation analysis. The Pearson's rank correlation coefficients (r) and

339 p -values of the correlations are included in Fig. 5a. As expected (Lee et al., 2007),
340 NDMA FP correlated poorly with bulk DOC ($r = 0.463$, $p > 0.05$) and DOC fractions
341 ($r = 0.427$ – 0.466 , $p > 0.05$, Pearson test). NDMA FP positively correlated with 3
342 $\text{kDa} < \text{MW} < 10 \text{ kDa}$ DON ($r = 0.709$, $p < 0.05$), which was stronger than with other
343 DON fractions ($r = 0.428$ – 0.662 , Pearson test, Fig. 5a), indicating that most of the
344 NDMA precursors were associated with the 3 $\text{kDa} < \text{MW} < 10 \text{ kDa}$ DON. This is in
345 marked contrast to the results of previous studies where most of the NDMA precursors
346 in municipal wastewater were of low molecular weight (Mitch and Sedlak, 2004;
347 Pehlivanoglu-Mantas and Sedlak, 2008; Qi et al., 2014). This difference may be due to
348 the characteristics of DBP precursors in industrial wastewater being quite different
349 from the DBP precursors in municipal wastewater (Lin et al., 2015). The DON, which
350 has an active ability to form NDMA, may be made up of influent pharmaceuticals and
351 personal care products (PPCPs). Previous researches have found that several PPCPs
352 could lead to the formation of significant amounts of NDMA (Roux et al., 2011; Shen
353 and Andrews, 2011). Five primary PPCPs (i.e., anastrozole, letrozole, epirubicin,
354 diclofenac, and bicalutamide) in wastewater along the WWTP treatment trains were
355 detected and quantified (Table S2 and Fig. S7 in the SI). However, no significant
356 correlation was observed between NDMA FP and the target PPCPs in this study ($p >$
357 0.05 , Pearson test). This indicates that some other specific PPCPs or nitrogenous
358 organic compounds are involved in the NDMA formation. Indeed, SMPs would
359 substantially increase NDMA FP (Nawrocki and Andrzejewski, 2011). Apart from

NDMA, the formation of other *N*-nitrosamines such as *N*-nitrosopyrrolidine (NPyr), *N*-nitrosopiperidine (NPip), *N*-nitrosodiethylamine (NDEA), *N*-nitrosomorpholine (NMor), and *N*-nitrosodi-*n*-butylamine (NDBA) from chloramination of surface water or wastewater has also been reported (Zhao et al., 2008; Yoon et al., 2013). The removal characteristics of NDMA FP were different from that of other *N*-nitrosamines in biological wastewater treatment (Yoon et al., 2013). To identify the DOM fractions responsible for the *N*-nitrosamines precursors in pharmaceutical wastewater, a future study on the statistical correlations between the DOC and DON fractions and the other *N*-nitrosamines FP is recommended.

3.3. Correlation of DON fractions and acute toxicity of DOM in pharmaceutical wastewater

The TU_a of DOM in pharmaceutical wastewater by luminescent bacteria assay and its correlation with the DON and DOC fractions are shown in Fig. 5b. Poor correlations were observed for TU_a of DOM and bulk DOC ($r = 0.180$, $p > 0.05$) and DOC fractions ($r = 0.037$ – 0.372 , $p > 0.05$, Pearson test). Therefore, in accordance with Köhler et al. (2006), DOC turned out to be not a good indicator of DOM acute toxicity in the industry wastewaters. A weak correlation was observed between TU_a of DOM and bulk DON ($r = 0.441$, $p > 0.05$), but TU_a of DOM showed a relatively good correlation with MW<3 kDa DON ($r = 0.659$, $p < 0.05$, Pearson test, Fig. 5b), thereby demonstrating that the MW<3 kDa DON fraction plays an important role in the acute toxicity of DOM. Notice that the TU_a value of DOM from MBBR effluent (S6) was

higher than 1.0 (Fig. S8 in the SI), the directly discharge of this biologically treated pharmaceutical wastewater to the receiving river may cause some acute toxicity to aquatic organisms (Wu et al., 2016). Thus, the MW<3 kDa DON may be candidates for future optimization processes, with the purpose of reducing the possibility of harm to aquatic organism. According to previous studies, low MW DON was difficult to remove by biological processes (Pehlivanoglu-Mantas and Sedlak, 2008; Huo et al., 2013). Consequently, effective tertiary treatment technologies are needed for the biologically treated pharmaceutical wastewater. Membrane technology (e.g., membrane bioreactor (MBR)), activated carbon, and advanced oxidation processes (e.g., UV/H₂O₂) have been applied to pharmaceutical wastewater treatment (see Gadipelly et al. (2014) for a review). However, previous studies have indicated that activated carbon and UV/H₂O₂ processes are ineffective in removing low MW DON (Chen et al., 2011a; Qi and Hu, 2016). MBR may improve the removal of low MW DON (Pehlivanoglu-Mantas and Sedlak, 2008), even though it has not been specifically used in the removal of DON in pharmaceutical wastewater.

3.4. Significance of the performance indicator of DON in pharmaceutical wastewater

Previous research has focused on DOC or TOC as a surrogate for DOM in pharmaceutical wastewater (Melero et al., 2009; Sirtori et al., 2009; Mascolo et al., 2010). However, DON also occurred in pharmaceutical wastewater, and proved to be an important fraction of DOM (Table 1). DOC removal is desirable because it reduces

the COD discharged to surface waters (Sirtori et al., 2009). However, those focusing on DOC or TOC for wastewater evaluation should also take into account further assessment parameters, such as the reduction of NDMA precursors and acute toxicity (Köhler et al., 2006; Yoon et al., 2013). A different conclusion is obtained when taking these additional parameters into account. For example, even though the removal of DOC is limited, MBBR and O processes were effective at removing the NDMA precursors (Fig. S8 in the SI). The Pearson correlation analysis indicated that the behavior of NDMA precursors and DOM acute toxicity was associated with the 3 kDa<MW<10 kDa and MW<3 kDa DON, respectively, and were not identical to that of the bulk DOC and DOC fractions (Fig. 5). In addition, the removal and molecular changes of DON are not coupled with that of DOC during biotreatment (Table 1, Fig. 4). Therefore, like DOC, DON and its fractions removal in pharmaceutical wastewater is also an important performance indicator.

4. Conclusions

This study investigated the removal characteristics of DON in pharmaceutical wastewater and its influence on the NDMA FP and acute toxicity of DOM. The major findings are: 1) Anaerobic and MBBR process were effective at sequestering DON, but the removal of DON by anoxic and aerobic processes was limited. 2) The removal and molecular changes of DON are not coupled with that of DOC during biotreatment. 3) The behavior of NDMA FP and acute toxicity of DOM was significantly associated with the 3 kDa<MW<10 kDa ($r = 0.709$, $p < 0.05$) and MW<3 kDa DON ($r = 0.659$,

$p < 0.05$), respectively, and were not identical to that of the DOC fractions ($r < 0.466$, $p > 0.05$, Pearson test). Therefore, measurement of DON and its fractions is recommended for a more comprehensive understanding of the DOM removal characteristics in pharmaceutical wastewater.

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Table 1 –DON, DOC, and the ratio of DON to DOC profile along the biotreatment units during three sampling events.

	S1	S2	S3	S4	S5	S6
DON profile (mg L⁻¹)						
Apr–2015	64.20 ± 4.45	54.25 ± 7.07	21.56 ± 1.79	20.38 ± 4.29	24.65 ± 1.75	10.20 ± 4.12
Jul–2015	70.58 ± 6.87	67.34 ± 8.75	19.29 ± 6.99	19.19 ± 0.99	19.02 ± 2.60	11.89 ± 0.64
Jan–2016	61.49 ± 5.71	54.26 ± 2.97	24.40 ± 4.37	24.11 ± 6.41	26.58 ± 1.34	14.11 ± 2.05
<i>p</i> –Value ^a	0.390	0.227	0.624	0.581	0.063	0.446
DOC profile (mg L⁻¹)						
Apr–2015	1824.53 ± 40.87	655.24 ± 32.67	177.35 ± 2.81	173.95 ± 3.51	127.27 ± 19.47	121.71 ± 9.04
Jul–2015	1782.12 ± 68.32	699.33 ± 39.85	200.11 ± 20.11	182.16 ± 3.01	163.24 ± 0.20	141.51 ± 26.63
Jan–2016	1999.84 ± 99.84	721.32 ± 15.88	241.22 ± 27.97	199.83 ± 44.82	141.22 ± 18.82	114.13 ± 16.08
<i>p</i> –Value ^a	0.113	0.244	0.104	0.641	0.214	0.428
DON/DOC profile						
Apr–2015	0.035	0.083	0.122	0.117	0.194	0.084
Jul–2015	0.040	0.096	0.096	0.105	0.117	0.084
Jan–2016	0.031	0.075	0.101	0.121	0.188	0.124

^a ANOVA *p* – value based on three sampling events.

Fig. 1. The biotreatment process for pharmaceutical wastewater and sampling points (S1–S6).

Fig. 2. The contribution of each tank to the total removal of DON and DOC during three sampling events.

Fig. 3. Van Krevelen diagrams showing all N-containing formulas in the mass spectra of (a) primary effluent (S1), (b) hydrolysis/acidification effluent (S2), (c) anaerobic effluent (S3), (d) anoxic effluent (S4), (e) aerobic effluent (S5), and (f) MBBR effluent (S6). Boxes overlain on the plots indicate the types of molecules modified from Antony et al. (2014).

Fig. 4. H/C vs H/C synchronous plots generated by applying 2D correlation on the subspectra of (a) CHON formulas and (b) CHO formulas. The correlation was based on the presence/absence of each individual formula along the treatment units. Red and blue colors indicate positive and negative correlation, respectively. White space indicates a lack of changes.

698

699 **Fig. 5.** (a) Pearson correlation between NDMA FP and DON and DOC fractions in
700 terms of: bulk, hydrophilic (HPI), hydrophobic (HPO), MW<3 kDa, 3 kDa<MW<10
701 kDa, 10 kDa<MW<30 kDa, and MW>30 kDa fractions. (b) Pearson correlation
702 between TU_a of DOM and DON and DOC fractions. A star indicates p -values < 0.05.
703 The observed correlations are available in Fig. S5 and S6 in the SI.