Production mechanism and characteristics of dissolved organic nitrogen derived from soluble microbial products (SMPs-DON) in a drinking water biological aerated filter
Jia Kang, Shu-li Liu, Teng-fei Ma and Xu Gao

ABSTRACT
Dissolved organic nitrogen derived from soluble microbial products (SMPs-DON) generated during the drinking water biological treatment process poses a great threat to water supply safety due to the potential carcinogenic risk. To further study the production mechanism and characteristics of SMPs-DON in drinking water biological aerated filtration (BAF), Illumina MiSeq sequencing is applied to characterize the microbial community. In addition, an excitation–emission matrix combined with the parallel factor model (EEM-PARAFAC) and gel filtration chromatography (GFC) are used to analyze the component and molecular weight (MW) distribution of the SMPs-DON. Results showed that the production of SMPs-DON in drinking water BAF can be explained using Illumina MiSeq sequencing from the perspective of the microbial community. Also, according to the EEM-PARAFAC analysis, the fluorescence intensity scores of fulvic-like and humic-like substances were almost unchanged, whereas the scores of protein-like substances first increased and then decreased, which was consistent with the variation in the DON concentration. SMPs produced initially primarily consisted of macromolecules with MW >20 kDa, and then they were degraded and small molecular SMPs with MW <5 kDa accumulated. This study provides theoretical guidance and technical support for ensuring drinking water safety and reducing secondary pollution risks from drinking water biological treatment.

Key words | component, dissolved organic nitrogen, molecular weight, production mechanism, soluble microbial products

INTRODUCTION
Soluble microbial products (SMPs) are produced accompanied by biomass growth, biomass decay, and substrate metabolism during biomass metabolism in the biological treatment process. The major chemical components of SMPs are humic and fulvic acids, polysaccharides, proteins, nucleic acids, organic acids, amino acids, antibiotics, steroids, extracellular enzymes, siderophores, cell structural components, and energy metabolic products, and others (Barker & Stuckey 1999). Among these components, humic substances, proteins, and carbohydrates have been proven to be the main components of SMPs (Shen et al. 2012). Dissolved organic nitrogen (DON) is a class of organic substances containing a series of nitrogen-containing functional groups such as various NH classes, amino categories, nitriles, purine, pyrimidine, and nitro compounds (Gu et al. 2010). SMPs released by the biomass in the process of organic matter degradation and biomass decay are rich in amino, amide and nitro functional groups, which are typical DON (Barker & Stuckey 1999).

The generation of SMPs not only lowers treatment limitations, but also produces a series of disinfection by-products (DBPs) with strong carcinogenicity during the disinfection process, which poses a major potential threat to drinking...
water safety. Moreover, a series of potentially more mutagenic and carcinogenic nitrogeous disinfection by-products (N-DBPs), such as haloenitromethanes (HNMs), haloacetonitriles (HANs), nitrosamines, and others, are produced from DON during disinfection, and HNMs have been listed as the highest priority for DBP control by the United States Environmental Protection Agency. Therefore, further research on the production mechanism and basic characteristics of DON derived from SMPs (SMPs-DON) in the drinking water biological treatment process will be helpful for improving effluent quality and reducing the safety risk of biological treatment.

The study of SMP production is a prerequisite for in-depth research of SMPs-DON. Yu et al. (2008) developed a theoretical model and an empirical model to predict the formation of SMPs during drinking water biofiltration. Carlson & Amy (2000) estimated the formation of SMPs in an ozone-biofilter by model prediction and direct measurement. Furthermore, previous studies have reported considerable variability in the production of SMPs in response to variation in microbial community structure. Tian et al. (2011) revealed that utilization-associated product (UAP) production in a membrane bioreactor during the autotrophy proliferation phase was lower than that in the heterotrophy proliferation phase. In a sequencing batch reactor, the fractions of UAPs produced by heterotrophs, ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB) were 78.5%, 14%, and 7.5%, respectively. Also, the fractions of biomass-associated products (BAPs) produced by heterotrophs, AOB, and NOB were 97.3%, 1.5%, and 1.2%, respectively (Xie et al. 2012). Moreover, heterotrophs can colonize some distance from autotrophs and still be sustained by autotrophic SMPs. Therefore, it is necessary to further study the formation mechanism of SMPs-DON by analyzing the microbial community structure.

Excitation–emission matrix (EEM) fluorescence spectroscopy can be correlated to the fluorescent components in SMPs-DON, thus giving more insight into the chemical composition and characteristics of SMPs-DON. However, DON presents various overlapping fluorophores in their EEM spectra, which makes the identification of dissimilar fluorescent groups difficult. A significant advancement was the introduction of the parallel factor (PARAFAC) model into the EEM analysis field. PARAFAC analysis successfully deconvolutes mathematically complex and spectrally overlapping EEM spectra into chemically independent components and allows for a more quantitative analysis of EEM spectra than visual inspection (Maqbool & Hur 2016). EEM-PARAFAC has been proven to be a suitable approach to get insight into the compositions, variations, and characteristics of dissolved organic carbon (DOC), DON, and SMPs in the drinking water biological treatment process (Yu et al. 2015).

The main objective of the current study is to further understand the production mechanism and characteristic variations of SMPs-DON during the drinking water biological treatment process. The production mechanism of SMPs-DON is explained from the perspective of microbiology by analyzing the relationship between SMPs-DON production and microbial community structure in a drinking water biological aerated filter (BAF). In addition, the componential characterization and the molecular weight (MW) distribution are investigated using EEM-PARAFAC and gel filtration chromatography (GFC), respectively.

**MATERIALS AND METHODS**

**Laboratory-scale BAF reactor setup**

Experiments were conducted in an upward flow laboratory-scale BAF made of plexiglass with an inner diameter of 50 mm and an effective working volume of 12.17 L. In the BAF reactor, the supporting layer was filled with pebbles, and ceramsite with a diameter of 2–5 mm was selected and filled in an 800-mm-high filter media layer. Sampling holes with diameters of 20 mm were equally distributed along the media layer at 16 cm intervals. A peristaltic pump (BT-100EA/153Yx*2PPS, Jieheng, China) was applied to maintain the filtration velocity at approximately 2 m/h. An air compressor (OLF-2530, Jiebao, China) was used to provide aeration and maintain the gas–water ratio in the range of 1.1–2:1. Backwashing was performed every other day. The composition of the synthetic source water was as follows to maintain the DOC concentration at approximately 20 mg/L and NH₄⁺–N concentration at approximately 10 mg/L: CH₃COONa, 62 mg/L; NH₄Cl, 38 mg/L; KH₂PO₄, 0.44 mg/L; K₂HPO₄, 0.56 mg/L; CaCl₂, 0.67 mg/L; MgSO₄,
0.67 mg/L; FeCl₃, 0.42 mg/L; CuSO₄, 0.0083 mg/L; KI, 0.05 mg/L; MnSO₄, 0.0333 mg/L; ZnSO₄, 0.0333 mg/L; CoCl₂, 0.0417 mg/L; NaMoO₄, 0.0067 mg/L. All the reagents used were of analytical grade. The influent pH was kept in the range of 7–8 by dosing with NaHCO₃. Both the COD and NH₄⁺–N removal efficiencies were higher than 65% before conducting this study.

**Water quality parameter measurements**

The collected water samples were filtered with pre-washed 0.45 μm cellulose acetate membranes (Anpel Co. Ltd, China), preserved at 4°C, and measured within 24 h. Total dissolved nitrogen (TDN) was measured using alkaline potassium persulfate digestion UV spectrophotometry. NO₃–N was measured using UV spectrophotometry. NO₂–N was measured using the N-(1-naphthyl)-ethylenediamine photometric method, and NH₄⁺–N was measured using salicylate–hypochlorite spectrophotometry. These experiments were conducted according to the Chinese National Standard Methods. DON was quantified as the difference between TDN and dissolved inorganic nitrogen (DIN): DON = TDN – NO₃– – NO₂– – NH₃/NH₄⁺.

**Microbial community structure analysis**

**DNA extraction, amplification, and Illumina MiSeq sequencing**

Microbial DNA was extracted from 10 to 15 g ceramsite using the OMEGA soil DNA extraction kit, according to the manufacturer’s protocols. DNA purity was detected using a NanoDrop2000 ultra-micro spectrophotometer (Thermo, USA). Polymerase chain reaction (PCR) amplification was conducted using a GeneAmp® 9700 PCR (Applied Biosystems, USA) and was performed in triplicate. Amplicons were detected using 2% agarose gels and recovered using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer’s instructions and quantified using QuantiFluor™-ST (Promega, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Majorbio, Shanghai) according to the standard protocols.

**Fluorescence EEM spectroscopy**

**EEM spectra determination**

Water samples of 100 mL were freeze-dried into powder, dissolved in 5 mL ultrapure water, and filtered with 0.45 μm cellulose acetate membranes (Anpel Co. Ltd, China). EEM spectra were determined using an F-7000 fluorescence spectrophotometer (Hitachi, Japan). The excitation and emission slits were both set to 5 nm, the scan speed was set at 12,000 nm/min, and the photomultiplier detector voltage was fixed at 700 V. The emission wavelength was increased from 220 to 600 nm at 5 nm steps by varying the excitation wavelength from 200 to 450 nm at 5 nm increments. Raman and Rayleigh scattering was removed by subtracting the EEM data of ultrapure water (as a blank sample) from that of water samples.

**MW distribution determination**

The MW distribution was determined through a GFC (Shimadzu LC-10AD, Japan) with a refractive index detector (Shimazu RID-10A). A TSKgel G4000PWXL column was used with Millipore water as the mobile phase at a flow rate of 0.5 mL/min. The column temperature was maintained at 40°C during operation. Water samples were filtered with 0.45 μm hydrophilic filtration membranes prior to injection (20 μL). MW calibration was achieved using EasiVial PEG/PEO (4 mL) standards for GPC/SEC (PL 2080-0200, Agilent Technologies) with MW ranging from 106 Da to 1,258 kDa.

**RESULTS AND DISCUSSION**

**Production mechanism of SMPs-DON in the BAF**

The profiles of DON concentration in the BAF are illustrated in Figure 1. The DON concentration in the BAF shows first an increase from 0.11 mg/L of the influent to 1.13 mg/L at the depth of 32 cm, and then a gradual decrease to 0.59 mg/L at the depth of 80 cm.

The microbial community structure analysis result in the BAF is presented in Figure 2. In the depth range of 0–32 cm,
the relative abundance of heterotrophic *Proteobacteria* exceeded 50%; the amount of SMPs-DON produced was greater than that of SMPs-DON degraded, and DON concentration increased. Thus, the variation of SMPs-DON was mainly attributed to the heterotrophic *Proteobacteria* within this depth range. As the filter layer depth increased to ≥48 cm, autotrophic *Nitrospirae* gradually increased and became the dominant bacteria. The degradation amount of the SMPs-DON was larger than the production amount, and the DON concentration gradually decreased. Within this depth range, the variation of SMPs-DON was mainly attributed to the autotrophic *Nitrospirae*.

The profile of DON in the BAF showed a trend of first increasing and then decreasing, while the relevant literature of Zhang et al. (2015) showed inconsistent results: they compared the concentration of DON in activated carbon, quartz sand, anthracite and ceramsite biofilters, and found that as the biofilter layer depth increased, the DON concentration first decreased and then increased. This is because biofilter media (Zhang et al. 2015), reactors (Lu et al. 2015), and other factors all have an effect on the generation and utilization of DON, and the relative importance between DON production and biodegradation varies in different reactors, even in different operation stages of the same reactor. In a biofilter for drinking water treatment studied by Liu et al.

![Figure 1](https://iwaponline.com/ws/article-pdf/19/7/1994/607702/ws019071994.pdf)  
*Figure 1* | Variations of TN, DIN, and DON concentrations in the BAF. (Error bars are standard deviations calculated from seven measurements.)

![Figure 2](https://iwaponline.com/ws/article-pdf/19/7/1994/607702/ws019071994.pdf)  
*Figure 2* | Relative abundance of bacteria phylum with different media depths of the BAF.
(2012), the normalized DON concentration \( \frac{C_{\text{effluent}}}{C_{\text{influent}}} \) after backwashing decreased from 0.98 to 0.90 in the first 1.5 h, and then gradually increased to approximately 1.5 in the following 8 h. Therefore the contradictory results were probably attributed to the production and biodegradability of DON.

**Component characterization of SMPs-DON by EEM-PARAFAC**

PARAFAC analysis was applied to decompose the fluorescence EEM spectra of all the SMP samples. One outlier sample was identified and removed from the dataset by discrete analysis, then CORCONDIA, as well as half-split analysis, was used to compute the optimal component number. PARAFAC models with two to seven components were computed, and the two-component model was validated by both analyses.

The contour plot, as well as the excitation and emission loadings for each component, is plotted in Figure 3. Component 1, whose peaks were located at Ex/Em of 245 nm/410 nm and 315 nm/410 nm, was similar to fulvic-like and humic-like substances from previous published studies (Ishii & Boyer 2012). Component 2 (Ex/Em: 225 nm/335 nm and 275 nm/335 nm) was associated with tryptophan-like proteins (Coble 1996) and protein-like substances associated with microbial activity and biological productivity (Seredyn'ska-Sobecka et al. 2011).

The fluorescence intensity scores of each component for samples at various media depths were also obtained using the PARAFAC analysis (Figure 4). Due to the difference in quantum efficiencies and individual responses to the...
quenching effects, it is not accurate to compare the relative concentrations of different components using fluorescence intensity scores (Baghoth et al. 2011). However, the fluorescence intensity scores are able to give an estimate of the relative concentration variation of each component. The scores of component 1 were nearly unchanged in the BAF, and fluctuated within the range of 8,570–8,762. In contrast, the scores of component 2 presented a trend of first increasing and then decreasing. The fluorescence intensity score rose from 5,629 of the influent to 7,484 at the depth of 16 cm, and then it was maintained at a high level in the depth range of 16–48 cm. After this, it dropped significantly to 4,893 at the depth of 80 cm. The variation in the fluorescence intensity score of component 2 was roughly consistent with that of the DON concentration. Therefore, DON variation in drinking water biological treatment processes can be referred to the protein-like fluorescence intensity score.

**MW distributions of SMPs-DON**

The GFC chromatograms of water samples from different media depths of the BAF were obtained (Supplementary Material, available with the online version of this paper), and the percentages in different MW ranges are shown in Figure 5.

The percentage of SMP MW in the range of <5 kDa increased from 0.10% to 35.62% as the filter layer depth increased from 16 to 80 cm, while the SMP fraction with MW >20 kDa decreased from 57.44% to 36.48%. The proportions of SMPs with MW in the range of 5–10 and 10–20 kDa were less affected by media depth, fluctuating in the range of 14.39–21.38% and 12.34–23.37%, respectively.

On the inlet side of the laboratory-scale BAF, the synthetic source water is metabolized by the biomass, and macromolecular SMPs are generated. As organic nutrients are depleted (the abundance of heterotrophic bacteria is gradually reduced), biodegradable SMPs generated in the previous stage are utilized by heterotrophic bacteria and converted into small molecular SMPs. Therefore, in the later stage of the drinking water biological treatment process, small molecular SMPs accumulate. In this study, SMPs initially produced primarily consisted of macromolecules with MW >20 kDa (0–16 cm), and then were degraded and small molecular SMPs with MW <5 kDa produced. Further inference indicated that an increasing hydraulic retention time (HRT) improved the removal efficiency of macromolecular organic matter in the drinking water biological treatment process, which was consistent with findings in the relevant literature (Aquino et al. 2009).

**CONCLUSION**

The production mechanism of SMPs-DON in drinking water BAF can be explained using Illumina MiSeq sequencing from the perspective of the microbial community distribution.

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**Figure 4** Variation of fluorescence intensity scores based on the PARAFAC model.

**Figure 5** Molecular weight distribution percentages of SMPs from different BAF media depths.
According to EEM-PARAFAC analysis, fulvic-like and humic-like substances were almost unchanged during BAF treatment, whereas protein-like substances first increased and then decreased, which was consistent with the variation in the DON concentration.

As the depth of the filter layer increased, SMPs with MW <5 kDa accumulated, while SMPs with MW >20 kDa decreased. Therefore, increasing HRT can improve the removal efficiency of macromolecular organic matter during the drinking water biological treatment process.

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