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Characterization of DON in IOM derived from M. Aeruginosa and its Removal by sunlight/immobilized TiO$_2$ system

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Abstract: Dissolved organic nitrogen (DON) is now considered as one of the most important precursors of nitrogenous disinfection byproducts (N-DBPs), and the algae cells were the main source of DON in eutrophic water sources. In this study, the characterizations of DON (especially proteins) in intracellular organic matter (IOM) were investigated. In addition, the DON removal by using sunlight/immobilized TiO$_2$ was also studied. The results showed that in algae cells, about 185 kinds of proteins were found and the distribution of isoelectric points of these proteins was mostly in acidic side. Apo-$\alpha$-phycocyanin and Apo-$\beta$-phycocyanin are the two main components, whose concentrations were 0.187mg/L and 0.136mg/L, respectively. The removal rate of DON by sunlight/TiO$_2$ system was about 29% after 7 hours’ irradiation, moreover, the system altered the molecular weight ranges of 30kD~80kD to the range of 20kD~30kD. Furthermore, proper photo-catalytic oxidation enhanced the coagulation efficiency significantly due to the direct removal and change of proteins.

Keywords: sunlight/TiO$_2$ oxidation; DON; IOM; proteins; coagulation

1. Introduction

Dissolved organic nitrogen (DON) is currently drawing more and more attention in drinking water treatment for its potential to form carcinogenic disinfection...
byproducts (DBPs). Chlorination and chloramination of DON not only produce
regulated DBPs such as trihalomethanes (THMs) and haloacetic acids (HAAs) but
also produce nitrogenous disinfection by-products (N-DBPs) such as nitrosamines,
halonitromethanes, and haloacetonitriles (Dotson et al., 2009; Lee et al., 2007;
Pehlivanoglu-Mantas and Sedlak, 2008). Toxicity tests have indicated that N-DBPs
are far more carcinogenic or mutagenic than some of the regulated DBPs (Plewa et al.,
2004; Richardson et al., 2007). DON is a complex mixture that is primarily composed
of amino acids, amino sugars, amides, peptides and heterocyclic-N compounds (e.g.
pyrimidine, imidazole, purine and porphyrins). However, a significant fraction of
DON originates from algae cellular lyses in eutrophic waters sources, which includes a
large spectrum of natural compounds like free and hydrolysable amino acids,
chlorophyll and amino-sugars, but also synthetic compounds like pesticides (e.g.
triazine) (Ambonguilat et al., 2006). Algae, including Microcystis aeruginosa, and
Chara, are widely studied in recent years (Li et al., 2008; Liu et al., 2014).
Pivokonsky (2006) has reported that a noticeable increasing concentration of proteins
is particularly evident at the cultivation of M. Aeruginosa, and the portion of proteins
included in IOM amounts up to 29.1% in their stationary phase. The proteins will
release into waters completely as the algae become dead, which account for the main
proportion of DON concentration. It has been reported that typical DON
concentrations in surface waters vary from less than 0.1 to higher than 10 mg N/L,
with a median value of approximately 0.3 mg N/L (Dotson and Westerhoff, 2009).
However, in eutrophic surface waters, DON concentration is generally higher, which
was found in the range of 1.0-2.0 mg N/L (Westerhoff and Mash, 2002, Pocernich and
Litke, 1997). Moreover, Bronk (2002) reported similar values for riverine waters (0.50 ± 0.28 mg N/L).
According to former studies, DON in IOM not only react with
oxidants/disinfectants (e.g., chlorine, chloramines, ozone (O₃) and potassium
permanganate) to form N-DBPs (Mitch et al., 2003; Richardson, 2003), but also
contributes to the membrane fouling (DiGiano et al., 2000; Her et al., 2000).
However, conventional water treatment processes (e.g., coagulation- sedimentation-
filtration) are ineffective in removing DON (Chu et al., 2011; Lee, 2005), and the remaining DON may be the precursors of N-DBPs during disinfection. Thus, removing DON efficiently is becoming a critical issue recently.

Algogenic organic matter (AOM) is involved in the reduction of coagulation efficiency (Cheng and Chi, 2003), and one of the inhibitory mechanisms is that the compositions of AOM can form complexes with cations in coagulant, which deteriorates the coagulation ability of the coagulant (Bernhardt et al., 1991). The proteins were viewed as the main inhibitory substances for the coagulation with polyaluminum chloride (PACl) and could consume PACl in the coagulation process due to the formation of chelate complexes between these inhibitory proteins and the coagulant (Takaara et al, 2007).

Photocatalytic oxidation using TiO₂ has raised more and more attention, because it’s widely available, non-toxic, inexpensive, and shows a relatively chemical stability. Additionally, the process can be carried out under wide conditions and leads to complete mineralization of organic carbon into CO₂, water and inorganic ion (Konstantinou and Albanis, 2004). Uyguner-Demirel (2011) reported the wide use of heterogeneous photocatalysis with TiO₂ (TiO₂/UV) to effectively remove NOM from water. However, limited information about removing DON by TiO₂ is available.

Therefore, the first object of this research is to investigate the characterization of DON in the IOM derived from M. Aeruginosa, then the effects and mechanism to remove DON by sunlight/TiO₂ system will be discussed. Additionally, the enhanced performance for coagulation by the oxidation of DON will also be confirmed.

2. Materials and methods

2.1 Materials

Reagents (e.g. Nessler reagent, coomassie brilliant blue) were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). All the chemicals used were at least of analytical grade, except as noted without further purification.
Microcystis aeruginosa which is a common species in eutrophic surface water was selected for this study. It was purchased from Institute of Hydrobiology, Chinese Academy of Sciences. Axenic cultures were carried out in batch mode in 1 L conical flasks with BG11 medium. The conical flasks were placed in an incubator and the algal cells were cultured at the temperature of 25°C with illumination of 5000 lx provided for 14h every day. The live algal suspensions were harvested with culture time between 15 and 28 days and diluted by ultra-pure water to prepare the EOM and IOM samples. The preparation method of EOM and IOM samples refer to our former study (Liu et al, 2011).

NOM obtained from International Humic Substances Society was dissolved by Milli-Q water and filtered through a 0.45-mm membrane to make a stock NOM solution.

2.2 Preparation and Characterization of immobilized TiO₂

TiO₂ was achieved by a conventional sol-gel method (Colon et al., 2006). Glass fiber nets were immersed in the sol for 5 min and dried under room temperature, finally calcined at 500°C for 2 h. While the above steps were repeated for 3-4 times, we could obtain immobilized TiO₂ particles supported on glass fiber nets.

The morphology and granularity of immobilized TiO₂ were examined by S4800 SEM (Hitachi, Japan). The photocatalyst constituents were identified by the combination of D8 Advance XRD (Bruker, Germany) and Ultra XPS (Kratos Analytical Ltd, UK).

2.3 Photo-degradation and coagulation experiments

For the sunlight/TiO₂ oxidation experiments, glass fiber nets loading TiO₂ were fixed on the inner wall of beakers, and immersed in 1000 mL water sample. The solutions were put outside simultaneously under sunlight irradiation for 7 h (9:30 am-16:30 pm), and continuously stirred during the reaction. Illumination intensity was measured and water samples were taken every 60 minutes. The samples were filtered
through 0.45 µm cellulose acetate membrane filters and placed in sample vials.

In coagulation experiments, the main steps followed that: certain dosages of polyaluminum chloride (PACl) and 1000 mL of each sample were put into a circular jar and agitated with a shaking apparatus at 150rpm for 30 min, following by a slow mixing at 100rpm for 20 min, and at 60rpm for 15 min. Samples were left for 30 min, then, each sample’s supernatant (100 mL) was collected by a U-shaped pipette in order to avoid the suction of precipitated solids. The samples were used to determine the turbidity, DON etc.

2.3 Analytical methods

All samples were filtered using 0.45 µm filters prior to chemical analysis. TOC were detected using a TOC analyzer (Multi N/C 2100, German). UV$_{254}$ was measured by a spectrophotometer (Shimadzu). DON was determined from the difference between measured total dissolved nitrogen (TDN) and sum of measured DIN species using Equation (1).

$$\text{DON (mg/L)} = \text{TN} - (\text{NH}_3 - N + \text{NO}_2 - N + \text{NO}_3 - N) \quad (1)$$

Three-dimensional excitation emission matrix fluorescence spectrophotometer was used to determine the composition of IOM (Hitachi F-4500 fluorescence spectrometer, Japan) following the procedures developed by Chen et al. (2003). Two-dimensional electrophoresis (2-DE) was used to determine the composition of IOM before and after oxidation, which carried out in a commercially available electrophoresis unit (GE Ettan DALT II system, GE, USA) according to published procedures (Bollag and Edelstein, 1991). Coomassie brilliant blue method is commonly used to determine the concentration of soluble protein, but it’s not adequate to fully investigate the evolution of protein. Based on 2-DE, Tandem Mass Tags (TMT) was used to identify the categories of main proteins.
3. Results and discussion

3.1 Characterization of DON derived from algae cells

Table 1 showed the DOC, DON, DOC/DON ratios and the SUVA values of IOM, the NOM and EOM were listed in the table as a comparison. The DOC/DON ratios followed the order: NOM>EOM>IOM. The much lower DOC/DON ratios of IOM indicated that they were rich in DON. Fig. 1 showed the EEM spectra of IOM solution. Coble et al. (1996) have reported that there are six fluorescence peaks: Peak A (humic acid substances), Ex/Em=237-260/400-500 nm; Peak B (tyrosine), Ex/Em=225-237/309-321 nm, 275/310 nm; Peak T1 (typtophan), Ex/Em=275/340 nm; Peak T2 (typtophan), Ex/Em=225-237/340-381 nm; Peak C (humic like substances), Ex/Em=300-370/400-500 nm; Peak M (marine humic substances), Ex/Em=290-310/320-410 nm. Referring to the fluorescence peaks and Fig. 1a, T1 and T2 typtophan are the main components in water samples. Similar results were gained at the study of Chen (Chen et al., 2003), which concluded that there were much the protein-like organic matter or org-N rich compounds in IOM. Similar EEM spectra picture was found for the pure phycocyanin (Fig.1b), which verified the above conclusions to some extent.

(a)                                 (b)

Fig. 1 Fluorescence EEMs of IOM and Phycocyanin (The IOM and Phycocyanin were diluted to the concentration was 1mg/L and 30mg/L, respectively )
Table 1 the properties of IOM, EOM and NOM

<table>
<thead>
<tr>
<th>NO</th>
<th>DOC(mg/L)</th>
<th>DON(mg/L)</th>
<th>DOC/DON</th>
<th>SUVA(mg/L/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOM</td>
<td>7.653</td>
<td>1.0</td>
<td>7.653</td>
<td>0.187</td>
</tr>
<tr>
<td>EOM</td>
<td>7.652</td>
<td>0.345</td>
<td>22.151</td>
<td>0.138</td>
</tr>
<tr>
<td>NOM</td>
<td>8.654</td>
<td>0.148</td>
<td>58.303</td>
<td>0.136</td>
</tr>
</tbody>
</table>

According to the former study (Fang et al, 2010), the concentrations of the measured free amino acids and aliphatic amines only accounted for 11.3% of the TON in IOM, while proteins in the algae cells were confirmed to be the inhibitory substances for the coagulation with PACI (Takaara et al, 2007). In addition, the free amino acids were easily transferred to inorganic nitrogen or utilized by the microbe in water. Protein and its derivations may be the predominate species of the DON in raw water that can interfere the performance of the subsequent water treatment processes. Therefore, it is essential to confirm the main protein categories and their feature in the IOM for the study of DON removal in drinking water treatment. Two-dimensional electrophoresis (2-DE) was used to determine the distribution of proteins in IOM and Tandem Mass Tags (TMT) was further used to identify the main categories of proteins in IOM. The results were shown in Fig. 2a and Table 2.

![Fig. 2 Two-dimensional electrophoresis of IOM (cDON=1mg/L).](image)

(a) : before photocatalytic oxidation; (b) : after photocatalytic oxidation
Table 2 Fluorescence intensity of untreated water samples

<table>
<thead>
<tr>
<th></th>
<th>Peak T1 tytophan</th>
<th>Peak T2 tytophan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex/Em=275/340nm</td>
<td>3764</td>
<td>4577</td>
</tr>
</tbody>
</table>

As seen from the electrophoresis spectra of IOM, about 185 protein spots were found in the spectra. The molecular weight (MW) of the main proteins was 30-80 kD and their isoelectric pH was mostly acidic. The results were in accordance with the MW distribution of organics in IOM to some extent (Liu et al, 2011). The particular proteins categories whose concentrations were higher than 0.066mg/L were further identified through TMT method (Table 3). There were about 15 kinds of proteins which were more than 0.066 mg/L in concentration and accounted for 1.519mg/L of total concentration. Apo-α-phycocyanin and Apo-β-phycocyanin were two main components, which were 0.187mg/L and 0.136mg/L in concentration, respectively. However, the concentration of the total dissolved proteins was about 7.22mg/L based on the Coomassie Brilliant Blue determination method, indicating that other proteins accounted for the residual concentration of the total proteins and may play an important role in the interference on the coagulation.

3.2 Characterizations of immobilized TiO$_2$ onto fiberglass cloth

It could be confirmed from Fig. 3 that the TiO$_2$ was stick to the fiberglass cloth tightly and the mean sizes of TiO$_2$ particles were about dozens of nanometers. The results of X-ray diffraction patterns showed that the TiO$_2$ stick on fiberglass was typical anatase TiO$_2$ and the mean sizes of TiO$_2$ particles were about 40nm (Fig. 4).
Fig. 3 SEM images of glass fiber nets
(a): glass fiber nets; (b) TiO$_2$ particles supported on glass fiber nets

Fig. 4 XRD diffractograms for TiO$_2$ catalysts.

3.3 Removal effects of DON and proteins

Fig. 5(a) and (b) showed the removal of DON and proteins by sunlight/TiO$_2$, respectively.
Fig. 5 Removal of DON and proteins by the solar/TiO$_2$ system

$(\rho$(DON) = 1.0 mg/L, pH = 6.0).

As seen from Fig. 5(a), sunlight/TiO$_2$ system could remove DON more effectively than the TiO$_2$ adsorption and sunlight degradation alone, which indicated that the reaction product between sunlight and TiO$_2$ may be responsible for the removal performance. It is well known that TiO$_2$ under the ultraviolet radiation could produce electron-hole pairs on its surface, then the electron-hole pairs react with water molecules to create OH radicals, which could oxidize most of the substance in the water, including org-N rich matters. Fig. 4b showed similar results for the removal of total dissolved protein in the IOM. As seen from Fig. 5a and b, in the same reaction condition, the removal rate of DON was significantly lower than that of total dissolved protein (29% Vs 48%). This could be explained by that DON represented the total nitrogen containing organics, including amino acids, proteins and other org-N, while the total dissolved protein only denoted the most categories of protein. The oxidation intermediates of proteins may also be other kinds of proteins or nitrogen containing organics, which also can be detected by DON.

As seen from Fig.2(a) and (b), photocatalytic oxidation using TiO$_2$ successfully degraded high MW (30kD~80kD) proteins into low ones (20kD~30kD). As shown in Table 3, the main categories of proteins of the raw IOM water samples were also found after oxidation, and the concentration decreased significantly. The concentrations of two typical proteins (Apo-α-phycocyanin and Apo-β-phycocyanin) were changed from 0.187mg/L and 0.136mg/L to 0.005mg/L and 0.009mg/L,
respectively. The removal effects gained 97.5% and 93.4%, which proved that both of the proteins were almost entirely oxidized. However, only a part of DON were directly oxidized to inorganic ion. To confirm the oxidation extent of the DON in water, the contents of total nitrogen, ammonium-nitrogen, nitrite-nitrogen, and nitrate-nitrogen were determined in the different reaction time. The results were shown in Fig. 6.

Fig. 6 The changes of TN, NO$_3^-$, DON, NH$_4^+$ in the DON degradation in the sunlight/TiO$_2$ system ($\rho$(DON) = 1.0 mg/L, pH = 6.0).

Seen from Fig. 6, the concentrations of TN and DON decreased at different speed while the concentrations of NH$_4^+$ and NO$_3^-$ increased to some extent, NO$_2^-$ wasn’t detected during the whole oxidation process. This indicated that parts of DON were oxidized to NH$_4^+$ and then to NO$_3^-$ through the oxidation of OH radicals and solar direct degradation. According to the results of Fig. 6, a small fraction of DON (less than 5%) was reduced from the solar direct degradation. However, little reduction of TN suggested that few nitrogen elements were transmitted to N$_2$ by the oxidative reaction and overflowed from the water. Based on the reported mechanism under UV and visible light irradiation by modified TiO$_2$ photocatalyst (Carvalho et al., 2010; Yoong et al., 2009) and oxidation of proteins and amino acids (Stadtman et al., 1993; Sante-lhoutellier et al., 2007), a photocatalytic mechanism is proposed as shown in Fig. 7.
3.4 Influence of enhanced coagulation by photocatalytic oxidation

The results of former study had concluded that AOM, especially IOM was involved in the reduction of coagulation efficiency (Cheng et al., 2003), and the main inhibitory mechanism was that AOM could form complexes with cations in coagulant, which deteriorated the coagulation ability of the coagulant (Bernhardt et al., 1991). Fig. 8 showed the influences of IOM before and after the oxidation on the coagulation performance.
Fig. 8 The influence of sunlight/TiO$_2$ oxidation on the coagulation with PACI ($\rho$(DON) = 1.0 mg/L, pH = 6.0)

Seen from Fig. 8, suspended kaolin was effectively coagulated with PACI and removed in the absence of IOM. On the other hand, the significant increase of supernatant turbidity was observed in the presence of IOM. However, a noticeable decrease of turbidity was observed when IOM was oxidized by sunlight/TiO$_2$ system. The supernatant turbidity for blank sample, IOM and IOM after oxidation were 0.5, 3.8, 0.85NTU, respectively. These results indicated that IOM brought about stronger coagulation inhibition, which was in accordance with the study of Takaara (Takaara et al., 2007). The oxidation process of the IOM would significantly decrease the inhibition effects, which might be caused by the variation of IOM composition, especially the kinds of proteins molecular. Pivokonsky (2006) has reported that AOM, such as proteins may capture aluminum and iron coagulant with a coordinate bond, which formed the complexes between metals and proteins, enabled the capturing of the coagulants and inhibited the coagulation process at drinking water treatment. Takaara (2007) also believed the cyanobacterial proteins could be one of the important causes of the increase in coagulant demand. As seen from Fig. 5, about 30% of DON and 50% of dissolved total protein were removed during the oxidation process, which could decrease the interference of IOM or protein to the coagulation performance. In addition, the drastic alteration in the molecular and property of the residual proteins in the sample could contribute to the reduction of disturbance for coagulation either. Fig. 2 and Table 3 showed the change of the protein molecular weights. According to Murphy (1990) and Makhataze (1995), hydrophobic interaction is the dominant force in protein folding and structure stabilization. To figure out the alteration on protein hydrophobicity, the hydrophobicity of IOM proteins before and after oxidation was investigated, and the results were shown in Fig. 9.
Fig. 9 showed that fluorescence intensity keeps increasing with reaction time, which demonstrates the enhancement of hydrophobic of protein. It may be due to the presence of OH radicals which destroyed the structure of protein, exposed hydrogen bond and aromatic amino acid side chain groups and thus increased the hydrophobic interaction of protein. The enhancement of coagulation efficiency may lead to the increasing hydrophobic of proteins. However, as the reaction proceeds, the concentration of DON decreased significantly, suggesting that decreased hydrophobic substances caused a decrease in coagulation efficiency.

As one of the main precursor for the N-DBPs, the removal effects of DON in the combined process by coagulation and sunlight/TiO2 oxidation at different reaction time were investigated. The results are shown in Fig. 10.
Fig. 10 The removal rate of DON removed by coagulation process and sunlight/TiO$_2$ system ($\rho$(DON) = 1.0 mg/L, pH = 6.0, PACl dosage = 20 mg/L).

Seen from Fig. 10, the removal efficiency of coagulation for DON increased initially and then decreased. However, the total removal effects for DON by the combined process almost kept constant after 2 hour’s oxidation, but the increase of removal efficiency accompanied by the decrease of coagulation efficiency. The removal rate of the combined process was about 40%. Proper oxidation time or suitable oxidation extents may be considered during the application in engineering. Many new catalysts were studied recently, which favored the absorption of visible and infrared solar photons (420-800 nm) and enhanced the oxidation effects under sunlight irradiation (Rtimi et al, 2015; Talebian et al, 2013; Xu et al, 2015). The enhancement of oxidation performance could strengthen the alteration of the proteins molecular, therefore lower reaction time may be needed, which could favor the real application.

4. Conclusion

4.1 The MW of proteins in IOM was 30~80 kD, and their isoelectric pH was mostly acidic. Apo-α-phycocyanin and Apo-β-phycocyanin are the two mainly components,
0.187mg/L and 0.136mg/L in concentration, respectively.

4.2 Immobilized TiO$_2$ was achieved by a conventional sol-gel method. SEM images indicated that TiO$_2$ nanoparticles were stick to the fiberglass cloth tightly. XRD revealed the anatase phase of obtained TiO$_2$. Moreover, sunlight/TiO$_2$ system can remove 29% of DON and 48.6% of proteins after 7 hours irradiation, and altered the molecular weight range of 30kD~80kD to the range of 20kD~30kD.

4.3 Photocatalytic degradation destroyed the protein structure, and caused an increase in the hydrophobic interaction of protein, which enhance the coagulation efficiency.

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