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Formation of water disinfection byproduct 2,6-dichloro-1,4-benzoquinone from chlorination of green algae

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ABSTRACT

We report that green algae in lakes and rivers can serve as precursors of halobenzoquinone (HBQ) disinfection byproducts (DBPs) produced during chlorination. Chlorination of a common green alga, *Chlorella vulgaris*, produced 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), the most prevalent HBQ DBP in disinfected water. Under varying pH conditions (pH 6.0–9.0), 2,6-DCBQ formation ranged from 0.3 to 2.1 μg/mg C with maximum formation at pH 8.0. To evaluate the contribution of organic components of *C. vulgaris* to 2,6-DCBQ formation, we separated the organics into two fractions, the protein-rich fraction of intracellular organic matter (IOM) and the polysaccharide-laden fraction of extracellular organic matter (EOM). Chlorination of IOM and EOM produced 1.4 μg/mg C and 0.7 μg/mg C of 2,6-DCBQ, respectively. The IOM generated a two-fold higher 2,6-DCBQ formation potential than the EOM fraction, suggesting that proteins are potent 2,6-DCBQ precursors. This was confirmed by the chlorination of proteins extracted from *C. vulgaris*: the amount of 2,6-DCBQ produced is linearly correlated with the concentration of total algal protein (R^2 = 0.98). These results support that proteins are the primary precursors of 2,6-DCBQ in algae, and control of green algal bloom outbreaks in source waters is important for management of HBQ DBPs.

Introduction

Surface waters are an important source of drinking water globally. While algal blooms are a regular seasonal occurrence in most surface waters, the extent and duration of these blooms is increasing due to anthropogenic causes (Yang et al., 2008; Zhang et al., 2010a, 2010b; Glibert, 2016). This poses a great challenge to the maintenance of drinking water quality, as algal blooms have been associated with clogged treatment plant pipes, odor and taste events, and toxicity events from toxin-producing blue-green algae (Knappe, 2004). Furthermore, increased algae and algal organic matter (AOM) in source waters has also been shown to affect the formation of drinking water disinfection by-products (DBPs) (Nguyen et al., 2005; Fang et al., 2010; Yang et al., 2011). AOM in source waters is derived from the metabolic activity of algae and is generally

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categorized as extracellular organic matter (EOM), products released by living cells, and intracellular organic matter (IOM), products of cell lysis (Li et al., 2012; Wert and Rosario-Ortiz, 2013).

The variation in biochemical composition between different species of algae and their fractions of AOM is well-documented, with noted differences between the numbers of unsaturated alkyl chains, carbohydrates, proteins, and other algal components (Brown et al., 1997; Pivokonsky et al., 2006). Thus, differences in DBP yield from algal precursors are often attributed to this biochemical variation and its resulting effect on DBP formation potential. For different phyla, *Nitzschia* sp. (diatom) showed higher chloroform yields (48 mg/mg C) but lower haloacetic acid (HAA) yields (43 mg/mg C) than *Chlamydomonas* sp. (green algae) (chloroform: 34 mg/mg C; HAA: 62 mg/mg C) and *Oscillatoria* sp. (blue-green algae) (chloroform: 26 mg/mg C; HAA: 72 mg/mg C) (Hong et al., 2008). In the same phylum, Huang et al. (2009) found that *Anabaena flos-aquae* yields were in the range of 2–11 μmol/mmol C for trihalomethane (THM) and 2–17 μmol/mmol C for HAA, while those of *Microcystis aeruginosa* were slightly higher.

Proteins or amino acids generally represent the largest fraction of the organic composition of many algal species, particularly cyanobacteria and green algae, with a protein fraction ranging between 70% and 36% on the basis of total dry weight (Kirpenko et al., 2016; Huang et al., 2016). Furthermore, the IOM fraction contains a significantly greater portion of proteins than the EOM. These differences also depend on the algal species, its growth phase, the age of the culture, and the culture conditions (Pivokonsky et al., 2006; Henderson et al., 2008). As a result, the presence of algae can dramatically affect the composition of amino acids in natural waters. Scully et al. (1988) examined the formation potential of THMs from chlorinated lake waters and found that algal proteins contributed to roughly 10% of the measured THM formation potential. Another study from Selbes et al. (2015) examined DBP formation from chlorination of nine amino acids and found that aspartic acid and histidine produced high amounts of dihalogenated haloacetonitriles (HANs) and HAAs. Thus, most studies that examined DBP formation potential from proteins and free amino acids have focused on the formation of C-DBPs (*e.g.*, THMs and HAAs) and certain N-DBPs (HANs and halonitromethanes (HNMs)) (Chu et al., 2010; Ge et al., 2011; Le et al., 2016). The relative contribution of different algal biochemical components to the formation of halobenzoquinone (HBQ) DBPs, however, has not been elucidated.

HBQs are an emerging class of DBPs predicted to be likely carcinogens on the basis of quantitative structure-toxicity relationship (QSTR) analysis (Qin et al., 2010; Bull et al., 2011; Yang and Zhang, 2013). The predicted chronic lowest observed adverse effect levels (LOAELs) for HBQs were estimated to be four orders of magnitude lower than those of the regulated THMs and HAAs. In addition, recent cytotoxicity and genotoxicity studies have shown that HBQs can cause DNA damage in T24 human bladder cancer cells and *Escherichia coli* (Du et al., 2013; Chen et al., 2015). Four HBQs were initially discovered as DBPs at levels ranging from 0.5 to 165 ng/L in drinking water (Zhao et al., 2012). These were 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), 2,3,6-trichloro-1,4-benzoquinone (TriCBQ), 2,6-dibromo-1,4-benzoquinone (2,6-DBBQ), and 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ). Among these four, 2,6-DCBQ is the most frequently detected HBQ and is found at the highest concentrations in both drinking water and recreational waters (Diemert et al., 2013; Wang et al., 2013a) Although phenol has been reported as a precursor (Zhao et al., 2012), the formation of HBQs from algal precursors has not been examined.

The objective of the current study is to determine whether algal precursors can produce HBQ DBPs during chlorination. *Chlorella vulgaris*, a commonly found green alga in surface water (Przytocka-Jusiak, 1984; Krienitz et al., 2015), was chosen as the target alga, and 2,6-DCBQ was selected as a typical HBQ due to its high occurrence frequency and abundance among HBQs (Zhao et al., 2010; Wang et al., 2014). Specifically, this study aims to (1) examine the formation of 2,6-DCBQ from *C. vulgaris* during chlorination; (2) compare 2,6-DCBQ yield from fractionated AOM (IOM and EOM fractions); and (3) determine the role of protein in 2,6-DCBQ formation.

1. Materials and methods

1.1. Materials

C. vulgaris (FACHB-6) was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. 2,6-DCBQ with a purity greater than 98% was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). Acetonitrile and formic acid (FA) (98%) were purchased from CNW Technologies (ANPEL Laboratory Technologies, Shanghai, China). Surrogate algal biomolecules were obtained as follows: bovine serum albumin (BSA) from AOBOX Biotechnology Co. (Beijing, China), fish oil (commercial Alaska fish oil) from Nu-Health Products Co. (Walnut, CA, USA), and starch from Tianjin Kemiou Chemical Reagent Co. (Tianjin, China). Stock solutions of chlorine were prepared by diluting a commercial solution of sodium hypochlorite (NaClO, 9% active chlorine). All other chemicals were reagent grade or higher and used without further purification. All solutions for this study were prepared with Milli-Q water (Mill-Q SP VOC; Millipore Co., Bedford, MA, USA).

1.2. Algae cultivation

C. vulgaris was maintained according to Organization for Economic Co-operation and Development (OECD) guidelines in OECD TG 201 media (pH 8.0) (OECD, 2011). The media contained the following: $NaHCO_3$ (50 mg/L), $FeCl_3{\cdot}6H_2O$ (64 μg/L), $CaCl_2{\cdot}2H_2O$ (18 mg/L), $MgSO_4{\cdot}7H_2O$ (15 mg/L), $MgCl_2{\cdot}6H_2O$ (12 mg/L), $Na_2EDTA{\cdot}2H_2O$ (0.1 mg/L), $ZnCl_2$ (3 μg/L), $CuCl_2{\cdot}2H_2O$ (0.01 μg/L), $MnCl_2{\cdot}4H_2O$ (0.415 mg/L), $CoCl_2{\cdot}6H_2O$ (1.5 μg/L), $Na_2MoO_4{\cdot}2H_2O$ (7 μg/L), H_3BO_3 (0.185 mg/L), NH_4Cl (15 mg/L), KH_2PO_4 (1.6 mg/L). All cultures were maintained in a SPX-250B-Z incubator (Shanghai Boxun Industry & Commerce Co., Shanghai, China) under a 12 hr light/12 hr dark regimen at 25 ± 0.5°C, with illumination provided by a 2500 lx fluorescent lamp. Algae samples for fractionation and for chlorination tests were collected during the exponential growth phase after four days of cultivation.

1.3. Extraction of EOM and IOM

The extraction of EOM and IOM was performed using a method modified from previously reported procedures (Wert and Rosario-Ortiz, 2013; Pivokonsky et al., 2014). After collection, cultivated algae were centrifuged at 10,000 r/min for 10 min. The supernatant was then collected and filtered through a 0.45 μm cellulose acetate membrane (Sartorius Stedim Biotech, Göttingen, Germany). The collected filtrate comprised the EOM fraction. The remaining pelleted cells were washed three times and re-suspended, all in Milli-Q water. To disintegrate the cell wall, the algal cells were subjected to three consecutive freeze/thaw cycles (–20°C/40°C) followed by 10 min of ultrasonic treatment (SCIENTZ-IID; Ningbo Scientz Biotechnology Co., Ningbo, China) at 30% power with 2 s of vibration at 3 s intervals. The IOM fraction was collected after filtration through a 0.45 μm cellulose acetate membrane.

1.4. Extraction of protein

To extract proteins from *C. vulgaris*, fresh IOM fractions were collected as described above (1.3 Extraction of EOM and IOM). The IOM fraction was precipitated in 50% ammonium sulfate at 4°C and recovered by centrifugation at 10,000 r/min for 10 min (Jiang et al., 2004). The precipitate was collected and dissolved in a small volume of phosphate buffer solution (pH 7.0) and dialyzed against the same buffer for 12 hr.

1.5. Chlorination tests

All chlorination tests were conducted in the dark under headspace-free conditions at a controlled room temperature (25 ± 1°C). Prior to chlorination, algal samples and extracted EOM and IOM fractions were analyzed for total organic carbon (TOC) using a TOC analyzer (TOC-LCPH; Shimadzu Co., Hong Kong, China). The chlorination of each sample used a ratio of Cl_2:TOC of 10 mg Cl_2/mg C (with starting dissolved organic carbon (DOC) of each sample at 30 mg/L) according to literature values (Liu et al., 2011; Liang et al., 2012) to maintain a residual $Cl_2 \geq 1$ mg/L. This was necessary for formation potential tests to ensure that the reactions were not chlorine limited. The pH of the reaction solutions was adjusted using HCl (0.01 mol/L) and NaOH (0.01 mol/L). The samples were kept oscillating throughout the reaction. Each sample (10 mL) was collected into an amber glass bottle and quenched with sodium thiosulfate (15 μmol). Samples were prepared in triplicate for DOC analysis (TOC-LCPH; Shimadzu Co.) or for analysis of 2,6-DCBQ, an orthogonal matrix experimental design was employed for the analysis of 2,6-DCBQ formation potential. The testing conditions for each of the three variables were: reaction time (2, 4, 6, 8, 12, 24, 48, 72, 96, and 120 hr), temperature (5, 15, 25, and 35°C), and pH (6.0, 7.0, 8.0, and 9.0). Chlorination of the surrogate algal biomolecules, protein (BSA), lipid (fish oil), and polysaccharide (starch), was conducted using the same conditions as for the algal fractions (25°C, pH 8.0, 24 hr). Chlorination of the extracted protein from *C. vulgaris* was also conducted under the same conditions (25°C, pH 8.0, 72 hr).

Two source water samples were collected using an organic glass hydrophore (2.5 L; Beijing Purity Instrument Co., Beijing, China) and transported to the laboratory on ice. The algae cells in each source water sample were collected and chlorinated at a ratio of Cl_2:TOC of 10 mg Cl_2 /mg C for 24 hr. The formation of 2,6-DCBQ was determined. The details of the water quality parameters, including Chlorophyll A content, are described in Table S1 (Appendix A).

1.6. Determination of 2,6-DCBQ by HPLC-UV analysis

The reaction solutions were centrifuged at 10,000 r/min for 5 min and filtered through a 0.45 μm cellulose acetate membrane to remove any particles prior to analysis. An Agilent 1260 series LC system consisting of a binary pump and an autosampler (Agilent, Waldbronn, Germany) with a Luna C18(2) column (100 × 2.0 mm i.d., 3 μm; Phenomenex, Torrance, CA, USA) was used for HPLC-UV analysis of 2,6-DCBQ at room temperature (25°C). The mobile phase consisted of solvent A (0.25% FA in water) and solvent B (0.25% FA in methanol) with a flow rate of 0.8 mL/min, with solvent A maintained at 40%. The detection wavelength was 273 nm, and the sample injection was 20 μL.

The experimental procedures adhered to strict quality assurance/quality control (QA/QC). Chlorine blanks, membrane blanks, and method blanks were prepared in triplicate and analyzed in parallel with the algal samples to control for any contamination during sample preparation. Each sample was analyzed in triplicate, and the average concentration and standard deviation were reported.

1.7. Confirmation of 2,6-DCBQ by HPLC–MS/MS analysis

An Agilent 6460 mass spectrometer coupled with an Agilent 1290 Infinity LC system was used for LC–MS/MS analysis of 2,6-DCBQ. Separation was achieved with a Luna C18(2) column (100 × 2.0 mm i.d., 3 μm; Phenomenex) at room temperature (25°C). A 10-port 2-position switching valve (Rheodyne, Rohnert Park, CA, USA) was used between the LC column outlet and the inlet of the MS to direct the LC effluent to the MS detector. The mobile phase consisted of solvent A (water containing 0.25% FA) and solvent B (acetonitrile containing 0.25% FA). The flow rate of the mobile phase was 0.3 mL/min, and the injection volume was 20 μL. The mobile phase gradient program began with 20% B; B was linearly increased to 90% over 20 min, and then maintained for 5 min; B was decreased to 20% over 25 min, and then maintained for an additional 4 min. The MS instrumental parameters were optimized as: gas temperature, 325°C; gas flow, 5 μL/min; nebulizer, 45 psi; sheath gas temperature, 350°C; sheath gas flow, 1 μL/min; capillary voltage, 3500 V; nozzle voltage, 500 V; fragmentor voltage, 70 V. The MS detector was operated in negative electrospray ionization (ESI) mode with multiple reaction monitoring (MRM). The limit of detection (LOD) for the method was determined to be 4.8 μg/L of 2,6-DCBQ. Further information regarding method validation is available in Fig. S1 and Fig. S2 (Appendix A).

1.8. Other analyses

Procedures for the analysis of the EOM and IOM fractions of *C. vulgaris* using Fourier transform infrared (FT-IR) spectroscopy and three-dimensional excitation-emission matrix (3D–EEM)

fluorescence spectroscopy are described in Appendix A, as are procedures for the analysis of Chlorophyll *a* content and the microscopic identification of algal species present in the source water samples. The concentration of sodium hypochlorite solution was standardized using iodometry. The pH of solutions was measured using a CyberScan pH 310 (Eutech Instruments, Singapore). The concentrations of polysaccharide and protein in the EOM and IOM fractions were determined by the phenol-sulfuric acid method and Bradford method, respectively (Zhang et al., 1999; Bradford, 1976). Each sample was analyzed in triplicate for each test.

2. Results and discussion

2.1. Formation of 2,6-DCBQ from chlorination of C. vulgaris

The formation of 2,6-DCBQ from chlorination of *C. vulgaris* under varying pH conditions (6.0–9.0) is shown in Fig. 1, determined using the HPLC-UV method. In general, the concentration of 2,6-DCBQ, expressed as μg per total mg of C, rapidly increased over the first 12 hr of chlorination, gradually reaching a plateau after 24 hr reaction time. This rapid increase is clearly observed in the reaction profile at pH 8.0 (Fig. 1), where the amount of 2,6-DCBQ produced nearly tripled from 0.6 to 1.7 μg/mg C from 2 to 12 hr chlorination time before plateauing at close to 2 μg/mg C after 24 hr of chlorination. No further increase in 2,6-DCBQ formation was observed when the reaction time extended past 24 hr under any of the tested pH conditions. These results demonstrate the significant influence of contact time on the formation of 2,6-DCBQ.

In Fig. 1, we also observed that 2,6-DCBQ formation from the chlorination of *C. vulgaris* is highly pH-dependent. 2,6-DCBQ

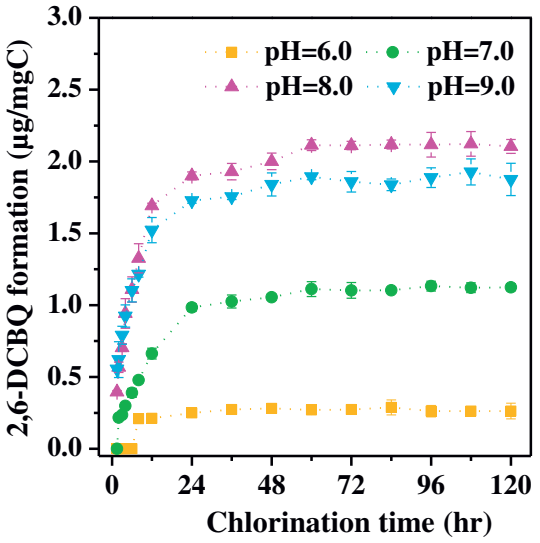

Fig. 1 – Formation of 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ) (μg/mg C ± SD; *n* = 3) from chlorination of *C. vulgaris* determined using an HPLC-UV method. Chlorination conditions: initial algal cell concentration = 30 mg/L as total organic carbon (TOC), chlorine:TOC = 10:1, temperature = 25 ± 1°C. The concentration of 2,6-DCBQ was calculated on the basis of 30 mg/L algal TOC.

concentrations were lowest at pH 6.0, while chlorination of the algae at pH 7.0, 8.0, and 9.0 significantly increased 2,6-DCBQ concentrations, with the maximum 2,6-DCBQ produced at pH 8.0. At 24 hr chlorination time, 2,6-DCBQ formation begins to plateau at 0.25 μg/mg C (pH 6.0), 0.99 μg/mg C (pH 7.0), 1.90 μg/mg C (pH 8.0), and 1.73 μg/mg C (pH 9.0), respectively. The formation of 2,6-DCBQ under optimized conditions was confirmed with HPLC-MS/MS (Appendix A Fig. S3). The observation that alkaline pH favored 2,6-DCBQ formation is similar to the formation of HAAs from the reactions of organics with chlorine (Deborde and von Gunten, 2008). This led to our hypothesis that alkaline pH levels result in the release of greater amounts of DOC from the algal cells.

The observed pH-dependent formation of 2,6-DCBQ from chlorination of *C. vulgaris* was further assessed via analysis of DOC in the reaction mixture present over chlorination time under the tested pH conditions (6.0–9.0) (shown in Appendix A Fig. S4). The DOC measurements represent the total AOM released from the cells during chlorination, which is a direct result of algal cell death and the subsequent decomposition of organic material in the solution. At pH 8.0, the DOC of the algae solution increased from 5 to 21 mg/L after 2 hr chlorination, while at pH 6.0, the DOC of the algae solution was only 8 mg/L after 2 hr chlorination. Throughout the measured reaction period (120 hr), total DOC was highest at alkaline pH conditions (pH 8.0–9.0) but lowest at acidic pH conditions (pH 6.0), consistent with the concentration of 2,6-DCBQ over chlorination time (Fig. 1). Thus, pH-dependent decomposition of *C. vulgaris* during chlorination results in variable amounts of DOC precursors for 2,6-DCBQ formation, with total DOC positively correlating with the amount of 2,6-DCBQ produced.

While pH conditions during chlorination can affect 2,6-DCBQ formation by increasing the availability of precursors, pH conditions can also directly influence 2,6-DCBQ formation *via* the reaction kinetics between AOM and chlorine. AOM is enriched in organic nitrogen in the form of proteins, amino acids, and amines (Fang et al., 2010; Li et al., 2012). HOCl can react with these organic compounds to produce organic chloramines (Deborde and von Gunten, 2008, Du et al., 2017; How et al., 2016; Li et al., 2010, 2011), an important intermediate in DBP formation, *via* the following second order reaction (Yoon and Jensen, 1993): $H_2NRCHCOOH + HOCl \rightarrow ClHNRCHCOOH + H_2O$.

The second order rate constants of chlorination reactions are typically dependent on pH, with the highest reaction rate achieved when the pH is one-half the sum of the pKa of the precursor and the pKa of chlorine (Deborde and von Gunten, 2008). Because the pKa values of most amino acids and fatty amines are around 10 (Hawkins et al., 2003) and the pKa value of HOCl is 7.5 (Ge et al., 2006), the reaction between AOM and chlorine is maximized in the pH range of 7.5–10. This is consistent with our findings, where the highest 2,6-DCBQ yield from chlorination of *C. vulgaris* was reached at pH 8.0–9.0 (Fig. 1).

In addition, we investigated the formation of 2,6-DCBQ from chlorination of algae in two source water samples collected from a water supply plant, one in June and one in October 2016. The two water samples were confirmed to contain *C. vulgaris* and other algae (Appendix A Fig. S5)and 5.2 and 18.9 μg/L Chlorophyll *a*, respectively (Appendix A Table S1). After chlorination, the June sample did not produce a detectable amount of 2,6-DCBQ, but the October sample contained 0.4 μg/mg C 2,6-DCBQ, which is

consistent with the higher Chlorophyll *a* in the sample. Thus, the formation of 2,6-DCBQ depends on algal concentration in source water, suggesting algae are important precursors of 2,6-DCBQ.

2.2. Formation of 2,6-DCBQ from chlorination of fractionated AOM

Next we identified the components of *C. vulgaris* AOM that contribute to the formation of 2,6-DCBQ. AOM was fractionated into IOM and EOM and separately chlorinated for 48 hr under varying temperature and pH conditions. The results (Fig. 2) show that 2,6-DCBQ formation from IOM and EOM is dependent on chlorination time, pH, and temperature. The formation of 2,6-DCBQ generally increased with higher temperature and was preferred at alkaline pH (8.0–9.0), with maximum 2,6-DCBQ produced at 35°C and pH 8.0 after 12 hr of chlorination (IOM: 1.81 μg/mg C; EOM: 1.06 μg/mg C). Interestingly, the amount of 2,6-DCBQ produced at 25°C, 15°C, and 5°C at alkaline pH peaked by 48 hr chlorination time. Thus, temperature and pH can strongly alter the kinetics of 2,6-DCBQ formation. However, because HBQs have been shown to transform to halo-hydroxyl-benzoquinones (OH-HBQs) over time in aqueous solution (Wang et al., 2014), it is important to note that the observed kinetics of 2,6-DCBQ formation in the reaction mixtures are likely influenced by both its formation and its degradation to OH-2,6-DCBQ.

Fig. 2 clearly shows that under the same testing conditions (pH 8.0, 48 hr chlorination time), the yield of 2,6-DCBQ from EOM was only 47.6% of that from IOM. Subsequent characterization of the IOM and EOM fractions revealed important differences between the two in terms of biomolecular composition, as presented in Table 1. The protein:DOC weight ratio within the IOM fraction was 0.2, nearly double the ratio within the EOM fraction at 0.1, suggesting that the IOM fraction is protein-rich (0.5 protein:polysaccharide ratio) in comparison to the EOM fraction (0.1 protein:polysaccharide ratio). In turn, the polysaccharide:DOC weight ratio of the EOM fraction (1.0) was twice that of the IOM fraction (0.5), indicating that the EOM fraction is polysaccharide-rich. The FT-IR spectra (Fig. 3) and 3D-EEM fluorescence spectra (Appendix A Fig. S6) confirmed this compositional difference. Interestingly, FT-IR analysis revealed the presence of lipid functional groups in the IOM fraction (peaks at 2926 cm^{-1} and 2853 cm^{-1}) that were absent in the spectra of the EOM fraction (Fig. 3). Thus, the difference in 2,6-DCBQ formation was likely due to the differing biomolecular composition of the fractions. This is consistent with studies of *M. aeruginosa*, where smaller quantities of C-DBPs and N-DBPs were produced from chlorinated or chloraminated EOM fractions than their corresponding IOM fractions, which was also attributed to higher amounts of amino acids (proteins) in the IOM fractions (Li et al., 2012). Wang et al. (2013b) also studied the

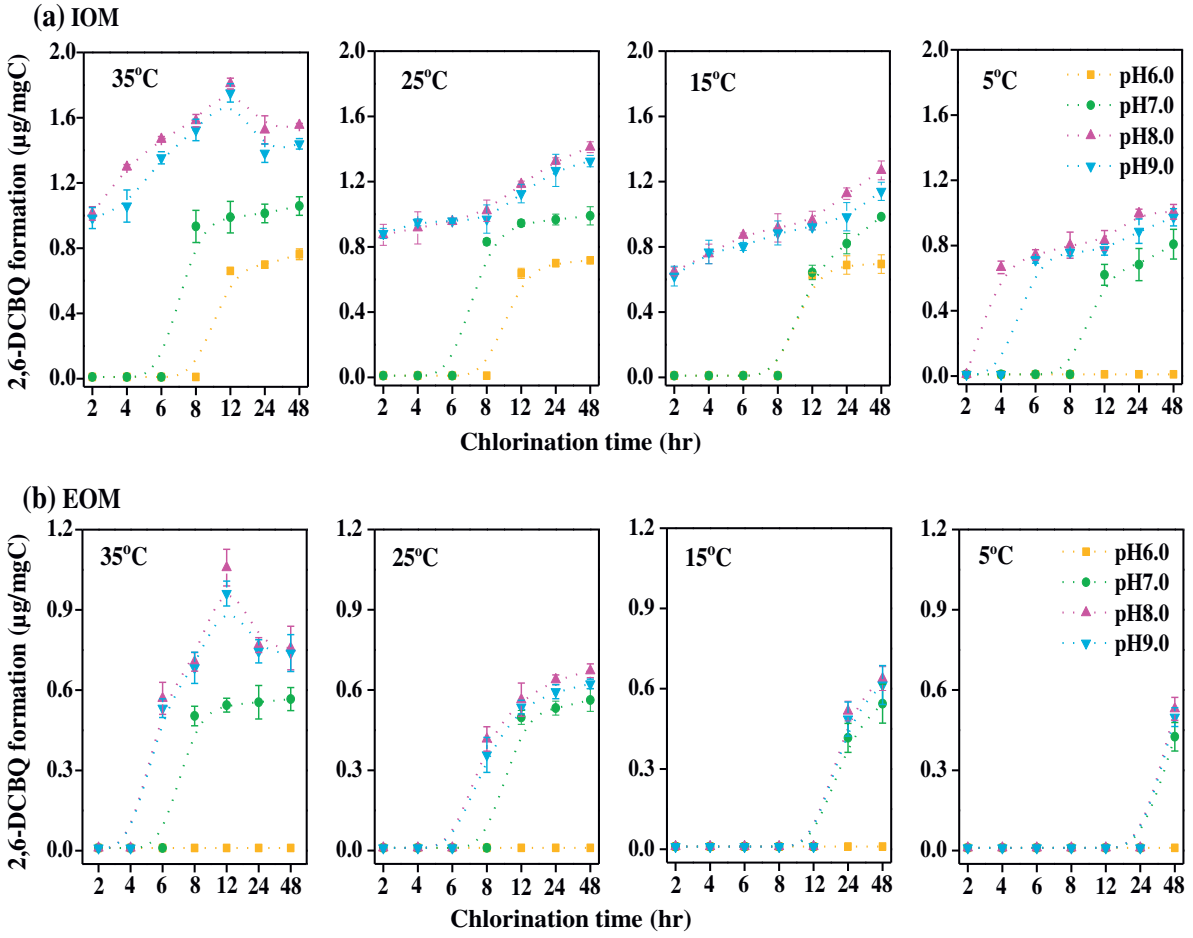

Fig. 2 – 2,6-DCBQ formation (μg/mg C ± SD; *n* = 3) from the chlorination of (a) intracellular organic matter (IOM) and (b) extracellular organic matter (EOM) fractions of *C. vulgaris* under variable pH and temperature conditions.

biochemical components of EOM from both *Pseudomonas aeruginosa* and *Pseudomonas putida* and found that DBP yield was much lower for polysaccharide monomers than for amino acids. These results suggest that algal proteins may be significant biomolecular precursors of 2,6-DCBQ.

2.3. Formation of 2,6-DCBQ from surrogate biomolecules and extracted protein

The differences in the biomolecular composition of the IOM and EOM fractions of *C. vulgaris* indicate the significant influence of biomolecules on 2,6-DCBQ formation, as higher 2,6-DCBQ production was found in the protein-rich IOM fraction, which also contained polysaccharides (Table 1; Fig. 3) and lipids (Fig. 3). To determine the biomolecular precursors of 2,6-DCBQ, the formation of 2,6-DCBQ was examined after chlorination of surrogate biomolecules representing algal protein, lipids, and polysaccharides. BSA and commercial fish oil were chosen as the surrogate compounds for algal protein and lipids because their amino acid composition and total polyunsaturated fatty acid content are similar to that of algal cells (Hong et al., 2008). It has also been observed that the principal form of carbohydrate in most algal cells (64%–96%) is polysaccharides, with glucose as the primary sugar (Brown et al., 1997). Hence, starch was selected as the surrogate. As shown in Table 2, 2,6-DCBQ formation was only detected from protein (BSA) precursors, producing up to 2.3 μg/mg C of 2,6-DCBQ at pH 8.0 after 24 hr chlorination. The surrogate polysaccharide and lipid biomolecules did not produce detectable 2,6-DCBQ under any of the tested chlorination conditions. These findings are consistent with previous studies which found that phenolic structures serve as the major precursors of HBQs (Zhao et al., 2012; Jiang et al., 2012). Hong et al. (2008) also demonstrated that proteins and lipids played a much more important role than polysaccharides in THM formation, while proteins were shown to be the dominant precursors of HAAs.

The results from the chlorination of surrogate biomolecules provides strong evidence that proteins are 2,6-DCBQ precursors. Thus, the role of algal proteins in 2,6-DCBQ formation was assessed through the examination of chlorinated *C. vulgaris* protein extracts. After chlorination, the amount of 2,6-DCBQ was found to correlate linearly with the amount of extracted total protein with a linear coefficient of $R^2 = 0.98$. Chlorination of the extracted total proteins from *C. vulgaris* produced as much as 1.9 μg/mg C of 2,6-DCBQ after chlorination for 48 hr (Fig. 4). Because the chlorination of whole cells of *C. vulgaris* produced 2.2 μg/mg C of 2,6-DCBQ (Fig. 1), it is likely that some insoluble cell material also contributes to 2,6-DCBQ formation.

Table 1 – Concentration (mean ± SEM; *n* = 3) of biomolecules present in the IOM and EOM fractions of *C. vulgaris*.

	IOM	EOM
DOC (mg/L)	10.0 ± 0.15	10.0 ± 0.15
Protein (mg/L)	2.3 ± 0.04	1.3 ± 0.06
Polysaccharide (mg/L)	4.9 ± 0.05	9.8 ± 0.07
Protein:polysaccharide (mg/mg)	0.5 ± 0.03	0.1 ± 0.03

IOM: intracellular organic matter; EOM: extracellular organic matter; DOC: dissolved organic carbon.

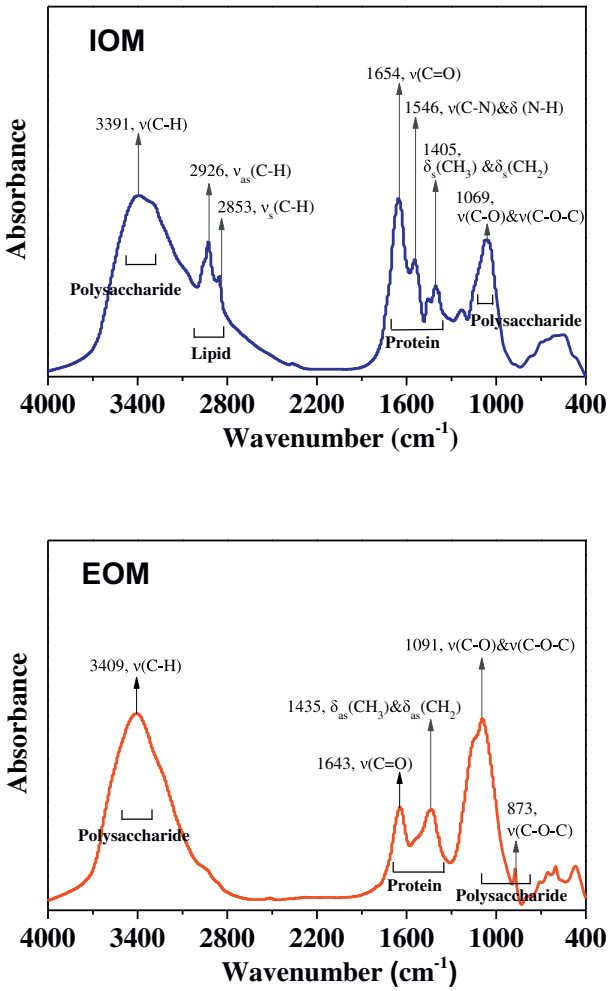

Fig. 3 – The FT-IR spectra of the IOM and EOM fractions from *C. vulgaris* showing the biomolecular composition of each fraction. Peaks associated with the presence of proteins (Giordano et al., 2001) (IOM: 1654 cm⁻¹, 1546 cm⁻¹,1405 cm⁻¹; EOM: 1643 cm⁻¹,1435 cm⁻¹) and polysaccharides (Jiang et al., 2012) (IOM: 3391 cm⁻¹, 1069 cm⁻¹; EOM: 3409 cm⁻¹, 1091 cm⁻¹, 873 cm⁻¹) were clearly detected in each spectra. Functional groups associated with the presence of lipids (Jackson and Mantsch, 1993) were also present in the IOM spectra (2926 cm⁻¹, 2853 cm⁻¹). ν_{as}, asymmetric stretch; ν_s, symmetric stretch; δ_{as}, asymmetric deformation (bend); δ_s, symmetric deformation (bend).

Table 2 – 2,6-DCBQ formation in the chlorination of surrogate biomolecules calculated on the basis of 30 mg/L DOC.

pH	Average formation of 2,6-DCBQ (μg/mg C ± SD; n = 3)		
	Carbohydrate	Lipid	Protein
6.0	n.d.	n.d.	0.7 ± 0.03
7.0	n.d.	n.d.	2.0 ± 0.01
8.0	n.d.	n.d.	2.3 ± 0.08
9.0	n.d.	n.d.	2.2 ± 0.04

Chlorination conditions: initial concentration = 30 mg/L as DOC, chlorine: DOC = 10:1, water temperature = 25°C, chlorination time = 24 hr.
n.d.: Not detected.

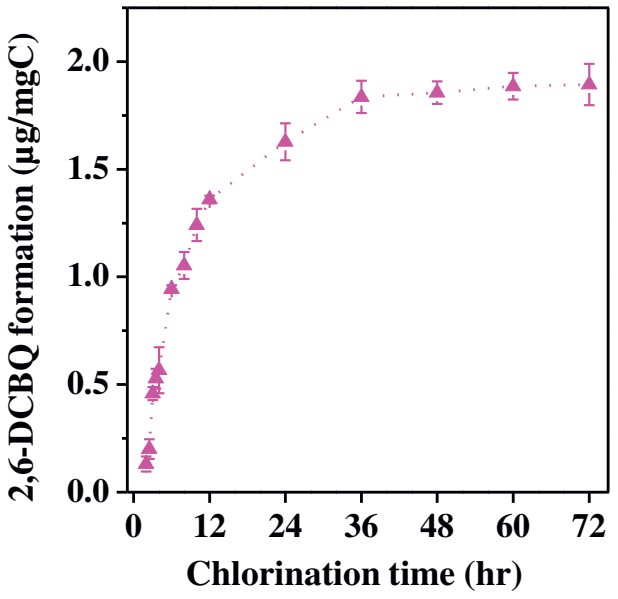

Fig. 4 – Formation of 2,6-DCBQ (μg/mg C ± SD; *n* = 3) from the chlorination of extracted total protein from *C. vulgaris*. Chlorination conditions: initial concentration = 30 mg/L as TOC, chlorine: TOC = 10:1, temperature = 25 ± 1°C. The concentration of 2,6-DCBQ was calculated on the basis of 30 mg/L TOC.

Overall, these results strongly support algal proteins as 2,6-DCBQ precursors from the chlorination of *C. vulgaris*.

3. Conclusions

C. vulgaris can produce 2,6-DCBQ, the most prevalent HBQ DBP, in disinfected water. Under varying pH conditions (pH 6.0–9.0), 2,6-DCBQ formation ranged from 0.3 to 2.1 μg/mg C with maximum formation at pH 8.0. The IOM fraction of *C. vulgaris* produces more 2,6-DCBQ than the EOM fraction. The proteins extracted from *C. vulgaris* were also confirmed as the primary biomolecular precursors of 2,6-DCBQ. Thus, green algae in lakes and rivers can serve as precursors of HBQ DBPs during chlorination, and control of green algal bloom outbreaks in source waters is important for management of HBQ DBPs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2017.10.001.

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