Environmental Pollution 219 (2016) 228–234

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Study on formation of 2,4,6-trichloroanisole by microbial *O*-methylation of 2,4,6-trichlorophenol in lake water☆

Kejia Zhang [a], Zhang Luo [a], Tuqiao Zhang [a], Minmin Mao [a], Jie Fu [b, *]

[a] College of Civil Engineering and Architecture, Zhejiang University, Hangzhou, 310058, Zhejiang, China
[b] Department of Environmental Science & Engineering, Fudan University, Shanghai, 200433, China

ARTICLE INFO

Article history:
Received 15 August 2016
Received in revised form
5 October 2016
Accepted 13 October 2016
Available online 28 October 2016

Keywords:
2,4,6-Trichloroanisole
2,4,6-Trichlorophenol
Chlorophenol *O*-methyltransferases
S-Adenosyl methionine
Control strategy

ABSTRACT

To explore the mechanisms and influence factors on the production of 2,4,6-trichloroanisole (2,4,6-TCA) in surface waters, the 2,4,6-TCA formation potential (FP) test was conducted by incubating the real lake water with the addition of 2,4,6-trichlorophenol (2,4,6-TCP) precursor. Besides bacteria and fungi, two common cyanobacteria and algae species, i.e., *Chlorella vulgaris* and *Anabaena flos-aquae*, have been proved to have strong capabilities to produce 2,4,6-TCA, which may contribute the high 2,4,6-TCA FP (152.2 ng/L) of lake water. The microbial O-methylation of 2,4,6-TCP precursor is catalyzed by chlorophenol *O*-methyltransferases (CPOMTs), and their characteristics were identified by adding inductive methyl donors or excluding microorganisms via ultrafiltration. The results indicated both *S*-adenosyl methionine (SAM) dependent and non-SAM dependent CPOMTs played important roles; extracellular CPOMTs also participated in the biosynthesis of 2,4,6-TCA. Moreover, investigating the effects of various environmental factors revealed initial 2,4,6-TCP processor concentration, temperature, pH and some divalent metal cations (i.e., Mn^{2+}, Mg^{2+} and Zn^{2+}) had obvious effects on the production of 2,4,6-TCA.

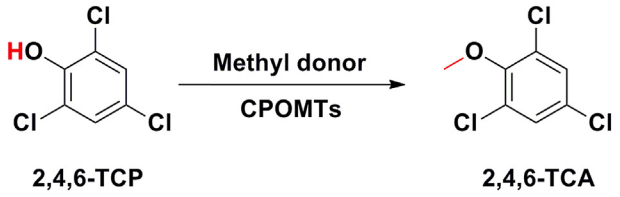
1. Introduction

Taste and odor (T&O) of the surface water supplies and especially of the drinking water reservoirs is a persistent problem for many water supply utilities (Fontana and Altamirano, 2010; Saito et al., 2008). The description of T&O most frequently cited by consumers is earthy-musty, which is mainly caused by several predominant compounds including 2-methylisoborneol (MIB, musty), geosmin (GSM, earthy), and 2,4,6-trichloroanisole (2,4,6-TCA, musty) (Jensen et al., 1994; Peter and Von Gunten, 2007). These T&O compounds are produced by microorganisms at nanogram per liter levels in the source water (Li et al., 2007). MIB and GSM in water have been associated with the presence of actinomycetes or their metabolic products, as well as with the presence of cyanobacteria and fungi (Dionigi et al., 1992; Jensen et al., 1994). 2,4,6-TCA is most probably formed by microbiological methylation of halophenols (Benanou et al., 2003). Due to low molecular weights and perpetual production, these T&O compounds are hardly removed by the water treatment process (such as coagulation, chlorination and sand filtration) (Bruce et al., 2002; Fu et al., 2017).

The T&O problem caused by chloroanisoles are well documented in drinking water and other food materials such as wines, eggs and poultry, chickens, pulp chips, dried fruits, and coffee (Coque et al., 2003; Curtis et al., 1972; Engel et al., 1966). 2,4,6-TCA is the dominant chloroanisole species and its perception threshold lowers than 4 ng/L (Benanou et al., 2003). Many bacteria (e.g., *Rhodococcus*, *Acinetobacter*, and *Pseudomonas* strains) and fungi (*Trichoderma* strains) have been reported to produce 2,4,6-TCA (Álvarez-Rodríguez et al., 2002; Coque et al., 2003). Whereas, fewer reports have been found on the production of 2,4,6-TCA by cyanobacteria and algae, which are common species in surface water. Studies indicate the dominant pathway for the biosynthesis of 2,4,6-TCA is the O-methylation of 2,4,6-trichlorophenol (2,4,6-TCP) precursor (Scheme 1) (Coque et al., 2003; Fontana and Altamirano, 2010). 2,4,6-TCP, a widely used fungicide, herbicide, insecticide and antiseptic (Ogunniyi et al., 2000), has been recognized as one of the greatest toxic environmental contaminants and frequently detected in surface, ground, and drinking waters (Halappa Gowda et al., 1985). A toxic test of 2,4,6-TCA on bacterium *Bacillus* sp. TL81 reported a half maximal inhibitory concentration (IC50) of 240 mg/L (Liu et al., 1982). The O-methylation process is

☆ This paper has been recommended for acceptance by Maria Cristina Fossi.
* Corresponding author.
E-mail address: jiefu@fudan.edu.cn (J. Fu).

http://dx.doi.org/10.1016/j.envpol.2016.10.042
0269-7491/



Scheme 1. Dominant pathway for biosynthesis of 2,4,6-trichloroanisole (2,4,6-TCA) from 2,4,6-trichlorophenol (2,4,6-TCP) precursor catalyzed by chlorophenol *O*-methyltransferases (CPOMTs).

thought to be a detoxification mechanism in various microbial species (Maggi et al., 2008), since 2,4,6-TCA is much less toxic relative to 2,4,6-TCP.

The microbial O-methylation process transfers a methyl group from donors to the hydroxyl group of 2,4,6-TCP via S_N2-like nucleophilic substitution, which is catalyzed by chlorophenol *O*-methyltransferases (CPOMTs). In term of methyl donors, CPOMTs can be divided into two types, i.e., *S*-adenosyl methionine (SAM) dependent and non-SAM dependent. The former CPOMTs use SAM as the methyl donor, while the latter can use a wide source of donors such as methanol, methylamines, and methanethiol. Currently, SAM dependent CPOMTs have attracted wide attentions and several 2,4,6-TCP induced enzymes have been purified from *Trichoderma longibrachiatum* (Álvarez-Rodríguez et al., 2002; Coque et al., 2003; Feltrer et al., 2010). In the nature waters, the nature organic material (NOM) may provide plentiful methyl sources for biosynthesis of chloroanisoles. Yet, the information on the non-SAM processes is still largely unknown.

In this paper, the formation potential (FP) of 2,4,6-TCA in different types of water (i.e., lake, river, and tap waters) was investigated with the addition of 2,4,6-TCP precursor. The lake water showed the highest FP of 2,4,6-TCA, and was then taken as the mode water to study the characteristic and mechanism of biosynthesis of 2,4,6-TCA in surface waters. The capability of typical microbial species for producing 2,4,6-TCA in the lake water was evaluated. The CPOMT types were identified through the induction of different methyl donors including SAM and other simpler molecules. The effects of various environmental factors including initial 2,4,6-TCP processor concentration, dissolved oxygen (DO) level, temperature, pH value, and metal cations on the production of 2,4,6-TCA were studied. The information can aid our understanding on the biosynthesis of T&O chloroanisoles in nature surface water, and making strategies on the control of 2,4,6-TCA.

2. Materials and methods

2.1. Chemicals

Reaction substances including 2,4,6-TCP, 2,4,6-TCA, SAM, and Coenzyme A (CoA) were of analytical grade (purity $\geq$ 98%), and purchased from the Sigma-Aldrich Corporation (St. Louis, Missouri, USA). 2-Isobutyl-3-methoxypyrazine (IB, $C_9H_{14}N_2O$, purity $\geq$ 99%), obtained from the Sigma-Aldrich Corporation, was used as the internal standard for 2,4,6-TCA analysis. A 100 mL of IB stock solution (20 μg/L) was prepared and stored in dark at 4 °C. For use in each time, a 20 ng/L of fresh IB solution was prepared by diluting the stock solution with ultrapure water produced by a Milli-Q apparatus (Millipore Corporation, Billerica, Massachusetts, USA). Organic solvents including methanol, ethanol, acetic acid, *n*-hexane, glycerin and other chemicals, such as sodium hydroxide (NaOH), hydrochloric acid (HCl), humic acid, sodium chloride (NaCl), etc. were of analytical grade, and purchased from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Of note, the lake water used in experiments was collected in the Qizhen Lake (Hangzhou, Zhejiang, China; 30°18′00″ N, 120°04′48″ E), the river water was collected in the Qiantang River (Hangzhou, Zhejiang, China; 30°12′36″ N, 120°10′12″ E), and the tap water was obtained from Zijingang Campus of Zhejiang University (Hangzhou, Zhejiang, China).

2.2. Experimental procedure

For a 2,4,6-TCA FP test, a general procedure was as follows: first, 0.2 mg/L of 2,4,6-TCP, which at the concentration limit of Standard for Drinking Water Quality (GB5749-2006), was added into water samples (i.e., lake, river or tap waters). Then, the solutions were incubated at 25 °C in an SPX-250B-Z thermostat incubator (Shanghai Boxun Industrial Co., Ltd., Shanghai, China) for seven days. Afterward, 100 mL of water sample was taken. After adding 30 g NaCl, the water sample was subjected to solid-phase microextraction (SPME) in a DK-S18 thermostat water bath (SENXIN Experimental Instrument Co., Ltd., Shanghai, China) at 65 °C for 30 min. The concentration of target 2,4,6-TCA was determined by gas chromatography-mass spectrometry (GC-MS) analysis.

To study the effect of initial precursor concentration, different concentrations of 2,4,6-TCP were added into the lake water. Anoxic/oxygenated lake water, prepared by aeration with nitrogen/oxygen gas, was used to evaluate the effect of DO level. Different incubation temperatures were applied to study the effect of water temperature. The pH of lake water was adjusted by NaOH/HCl to explore the effect of matrix pH. Different common metal cations were added into the lake water to assess their impacts on the biosynthesis of 2,4,6-TCA. To characterize the CPOMTs, different methyl donors including SAM, *n*-hexane, ethanol, methanol, glycerol and humic acid, and cofactor CoA were added into the lake water to determine their impacts on the biosynthesis process. The ultrafiltration was subjected to lake water to identify the contributions of intracellular and extracellular CPOMTs. In addition, the common microbial species in the lake water were identified and evaluated their capabilities for producing 2,4,6-TCA by using the same procedure as described above. Four strains, *Escherichia coli* BNCC133264, *Cyclotella hebeiana* FACHB-1030, *Chlorella vulgaris* FACHB-1227, and *Anabaena flos-aquae* FACHB-1092 were provided by either the BeNa Culture Collection (Beijing, China) or the Freshwater Algae Culture Collection of the Chinese Academy of Sciences (Wuhan, China). All the FP tests were conducted in duplicate and average values were reported in this paper.

2.3. Experimental analysis

An inoLab Oxi 730 oxygen meter (WTW GmbH Company, Weilheim, Germany) was used for detecting the DO level in water. The total oxygen carbon (TOC) of water samples were measured using a TOC-VCPH analyzer (Shimadzu Corp., Kyoto, Japan). The concentration of 2,4,6-TCA was determined using SPME followed by GC-MS analysis. A manual SPME handle and 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco Inc, Bellefonte, Pennsylvania, USA) were used for extraction. The detailed procedure was described elsewhere (Zhang et al., 2015). A QP2010 Plus GC-MS instrument (Shimadzu Corp., Kyoto, Japan) equipped a RTX-5MS column (30.0 m × 0.25 mm × 0.25 μm) was used to analyze 2,4,6-TCA. The GC carrier gas was high purity of helium (>99.99%) with a pressure of 90 kPa and a flow rate of 1.45 mL/min. The sample injection adopted splitless mode and the inlet temperature was 250 °C. The initial column temperature was 60 °C (held for 3 min), then heated to 150 °C by 6 °C/min, and finally heated to 250 °C by 15 °C/min (held for 3 min). An electron ionization (EI) source with an energy of 70 eV was used for MS