Effects of 2,2-dichloroacetamide (DCAcAm), an emerging disinfection by-product in drinking water, on the intestinal microbiota of adult zebrafish

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ABSTRACT

The presence of disinfection by-products (DBPs) increases the mutagenicity of water and may pose adverse health effects. Gut microbiota exerts a fundamental role on host physiology, and how extrinsic perturbations influence its composition has been increasingly examined. However, the effect of DBPs on gut microbiota is still poorly understood. In the present study, adult zebrafish were exposed to different concentrations of dichloroacetamide (DCAcAm, an emerging nitrogenous DBP) for 30 days. Sequencing of 16S rRNA amplicons revealed a significant change in the richness and diversity of microbiota in the gut of DCAcAm-exposed zebrafish. At the phylum level, the abundance of Proteobacteria decreased and the abundance of Fusobacteria and Firmicutes increased significantly in the gut after exposure to 100 and 500 μg/L DCAcAm. At the genus level, the abundances of several bacteria which are considered pathogens or opportunistic pathogens in fish and closely related to fish metabolism, disease and inflammation (*Aeromonas*, *Stenotrophomonas*, *Bacteroides* and Ralstonia) increased in the DCAcAm-treated groups. Our results reveal that DBPs in drinking water potentially affect gut microbiota composition, which may contribute to the toxicity assessment of DBPs in future and provide new insight into the complex interactions between the DBPs in drinking water and host health.

Key words | dichloroacetamide, disinfection by-products, gut microbiota, zebrafish

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INTRODUCTION

The disinfection of drinking water has been hailed as one of the most important advances ever for protecting public health (Ngwenya *et al.* 2013). While the efficacy of chemical disinfectants in controlling pathogens is well recognized, one of the main concerns is the reactivity of disinfectants with organic and inorganic substances to generate harmful disinfection by-products (DBPs) (Yang *et al.* 2019). Research into DBPs dates back to the discovery of trichloromethane in the 1970s and since then, numerous studies have been conducted to increase the understanding of DBPs in terms of their formation processes and exposure levels in drinking water distribution systems and the associated human health effects (Li & Mitch 2018; Mian *et al.* 2018). To date, around 700 DBPs have been identified in drinking waters, and the possible population health impacts associated with exposure to emerging DBPs are of particular interest due to their high cytotoxicity, genotoxicity, developmental toxicity and potential carcinogenicity (Cortes & Marcos 2018; Han & Zhang 2018). Accumulating evidence demonstrates that exposure to DBPs is associated with the increased risks of birth defects and a number of diseases such as colon, colorectal, rectal and bladder cancer (Rahman *et al.* 2014; Hrudey *et al.* 2015; Han *et al.* 2017; Jiang *et al.* 2017). In recent years, haloacetamides (HAcAms) in drinking water have

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raised wide concerns due to their high toxicity compared with the regulated carbonaceous DBPs (Bond *et al.* 2011; Yang *et al.* 2014). Dichloroacetamide (DCAcAm), the most frequently detected member of HAcAms, were approximately two orders of magnitude more cytotoxic than dichloroacetic acid (Huang *et al.* 2012). Thus, the risk of DCAcAm for humans via long-term water drinking cannot be ignored.

Gut microbiota, the large number of microbial species residing in the gastrointestinal tract, is increasingly known to play a pivotal role in the maintenance of host health (Abdollahi-Roodsaz *et al.* 2016; Cani 2018). It has been established that changes in the gut microbiota are associated with not only obesity, diabetes and liver diseases but also cancer and even neurodegenerative diseases (Barichella *et al.* 2018; Yoshida *et al.* 2018). Furthermore, gut microbiota dynamics are highly sensitive to exogenous stressors. A few recent studies have demonstrated that exposure to different kinds of environmental pollutants, including antibiotics, nanomaterials, pesticides, heavy metals and persistent organic pollutants, can effectively induce gut microbiota dysbiosis in different experimental models (Kan *et al.* 2015; Jin *et al.* 2017; Chen *et al.* 2018a, 2018b). However, information about the effects of DBPs on the intestinal microbiota remains limited.

Considering the prevalence and potential adverse human health effects of DBPs in disinfected drinking water, we wondered whether the 'DBPs' associated with the 'gut microbiota'. For this purpose, we exposed adult zebrafish to various concentrations of DCAcAm, an emerging nitrogenous DBP that was widely detected in disinfected drinking water, and determined whether or not they could induce microbiota dysbiosis in the gut by using 16S rRNA gene deep sequencing. The results acquired in the present study may contribute to the current knowledge of the link between DBPs and intestinal microbiota, and provide new information regarding DBPs-induced toxicity.

METHODS

Chemicals and reagents

The DCAcAm was obtained from Alfa Aesar (Karlsruhe, Germany). A stock solution of DCAcAm was prepared in dimethyl sulfoxide of high-performance liquid chromatography-grade (DMSO; Sigma-Aldrich, USA). All other reagents were of analytic grade.

Fish maintenance and exposure

Adult zebrafish were purchased from the China Zebrafish Resource Center. Each fish was raised in a separate tank with 300 mL charcoal-filtered and 24-h aerated tap water at a constant ambient temperature of $28.0 \pm 2.0\,^{\circ}C$ under a light/dark cycle of 14/10 h. The fish were fed twice daily with commercial flake food (Sera Vipan, Petco, USA). After 2 weeks' acclimation, fish were randomly divided into four groups (10 fish in each group), including a control group (0 μg/L DCAcAm) and the three DCAcAm-exposure groups (10, 100 and 500 μg/L DCAcAm). The control group received an equal volume of DMSO alone. The final DMSO concentrations in the control and exposure groups were <0.001%. The water was renewed daily, to maintain the appropriate concentrations of DCAcAm. After 30 days of exposure, the zebrafish were anesthetized in 0.03% MS-222 (Sigma-Aldrich) and dissected for their intestines, which were immediately snap-frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ for the following microbiota analyses.

Measurement of DCAcAm

The actual concentrations of DCAcAm in exposure media after renewal were measured according to a previous method with a small modification (Yu *et al.* 2015; Lin *et al.* 2016). Briefly, the extraction of DCAcAm was performed using liquid–liquid extraction with ethyl acetate. The detection was carried out using an Agilent 7890A capillary gas chromatograph coupled with a micro-electron capture detector. DCAcAm was separated via DB-5MS column (30 mm × 0.25 mm × 0.25 μm, Agilent, USA).

16S rRNA amplicon sequencing and bioinformatic analyses

There are a series of procedures from DNA extraction to final data acquisition including total DNA extraction,

polymerase chain reaction and product purification, library construction, sequencing and data analysis. All procedures were conducted using Novogene Bioinformatics Technology Co. Ltd (Beijing, China).

Briefly, after each exposure phase, nine zebrafish in each group were randomly chosen for gut microbial community analysis. From each group, three intestines excised from three individual zebrafish were pooled together and considered a biological replicate ($n = 3$ per treatment group). Total bacteria DNA was extracted from intestinal samples using the cetyltrimethylammonium bromide method. The purity and concentration of DNA extraction were determined by electrophoresis on 1% agarose gels. Genomic DNA were then used for the amplification of 16S rRNA using the primer pair 341F (50-CCTAYGGGRBGCAS CAG-30) and 806R (50-GGACTACNNGGGTATCTAAT-30), targeting the V3–V4 hypervariable regions. Then, the amplicons were sequenced on an Ion S5^{TM} XL platform and 400 bp/600 bp single-end reads were generated. The raw reads were filtered according to the cut adapt quality controlled process (V1.9.1, http://cutadapt.readthedocs.io/en/stable/) and chimera sequences by use of UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) (Edgar *et al.* 2011; Haas *et al.* 2011). The effective tags were then clustered by UPARSE (Uparse v7.0.1001 http://drive5.com/uparse/) to operational taxonomic units according to 97% similarity. After taxonomic annotation based on the RDP classifier (version 2.2) and the Green Genes Database, the number of sequences was calculated and summed for each classification level (e.g., phylum and genus) to compare the gut microbial abundance and diversity among exposure groups. Alpha- and beta-diversity analyses were conducted with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). After rarefying the number of reads, observed-species, Chao1, Shannon, Simpson, ACE and Good's coverage were calculated, and statistical significance was determined using Student's *t*-tests and a 5% false-discovery rate. Principal coordinate analysis (PCoA) was displayed by WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3) to get principal coordinates and visualize from complex, multidimensional data.

RESULTS AND DISCUSSION

Concentration of DCAcAm in the exposure media

Nitrogenous DBPs in drinking water, especially the emerging HAcAms which have demonstrated higher cytotoxicity and genotoxicity than carbonaceous DBPs and other nitrogenous DBPs (e.g., haloacetonitriles), have limited study in terms of their formation, removal and toxicity properties and thus are a cause of wide concern (Bond *et al.* 2011). Among the detected HAcAms, DCAcAm is present at the highest concentration and caused significant chronic cytotoxicity and acute genotoxicity (Huang *et al.* 2012). In this study, DCAcAm-induced changes in gut microbiota composition of adult zebrafish were characterized to provide a new perspective on the investigation of DBPs' toxicity. A low treatment concentration of DCAcAm (10 μg/L) was selected to represent DCAcAm exposure from oral intake of drinking water (Cortes & Marcos 2018), while the medium treatment (100 μg/L) represents those of fish with high contents of organic nitrogen which can serve as

Table 1 | Alpha diversity in zebrafish intestines after DCAcAm exposure (0, 10,100 and 500 μg/L)[a]

Group	Observed species	Shannon[b]	Simpson[b]	Chao1[c]	ACE[c]	Good's coverage (%)[d]
0	717 ± 154	4.38 ± 0.76	0.78 ± 0.10	765.37 ± 182.24	772.69 ± 179.85	99.87 ± 0.06
10	412 ± 85*	2.70 ± 0.38*	0.66 ± 0.07	463.02 ± 86.14	468.24 ± 90.80	99.90 ± 0.01
100	425 ± 65*	3.13 ± 0.22	0.74 ± 0.06	483.94 ± 71.48	488.76 ± 39.38	99.87 ± 0.06
500	515 ± 98	3.48 ± 0.37	0.72 ± 0.07	576.46 ± 91.75	566.81 ± 89.90	99.90 ± 0.00

[a]Values represent the mean ± SD of replicates.
[b]Indicative of bacterial community diversity.
[c]Indicative of bacterial community richness.
[d]Indicative of bacterial sequencing coverage.
*$P < 0.05$ indicates significant difference between DCAcAm exposure groups and the control group.

a source of DCAcAm precursors, and an additional high DCAcAm exposure (500 μ/L) for fish in polluted systems. Concentrations of DCAcAm in each DCAcAm exposure group were detected to be 9.82 ± 0.21 μg/L, 100.43 ± 0.82 μg/L and 498.96 ± 1.48 μg/L, respectively. No DCAcAm was found in the control group. Concentration used in the present study was an environmentally realistic one; hence, there is a guarantee of greater ecological relevance from the results based on the current data.

Overall characterization of the sequencing data

Given the essential role of the gut microbiome in a variety of aspects of human health coupled with the potential health risk of DCAcAm, there is a need to elucidate the effects of DCAcAm exposure on the gut microbiome. Although DCAcAm has been demonstrated to cause the acute metabolism and DNA damage in zebrafish (Lin *et al.* 2016), toxicological literature concerning the effects of DCAcAm on gut microbiota of zebrafish is scarce. To the best of our knowledge, only one similar study to date has demonstrated that the diversity of the gut microbiome in mice was significantly reduced due to trichloroacetamide exposure. Consistent with our findings, a number of perturbed gut microbes such as Firmicutes, Bacteroidetes and Proteobacteria were significantly changed (Zhang *et al.* 2015).

In the present study, adult zebrafish were exposed to different concentrations of DCAcAm for 30 days. The summary information of pyrosequencing of the V3–V4 region of 16S rRNA genes is shown in Table 1. The values of Good's coverage were nearly 100% for all sequences in the four groups, suggesting sufficient sequencing depth for the investigation of gut microbiota. There were differences between the control and DCAcAm-treated groups (10 and 100 μg/L) regarding the number of species detected (Figure 1(a)). The Shannon diversity index also suggested that the diversity of the gut microbiota was influenced by 10 μg/L DCAcAm exposure (Figure 1(b)). No significant differences were observed among DCAcAm treatment groups. In addition, PCoA results showed that communities from different DCAcAm exposure groups were clearly more distant, particularly from those of the control group (Figure 1(c)).

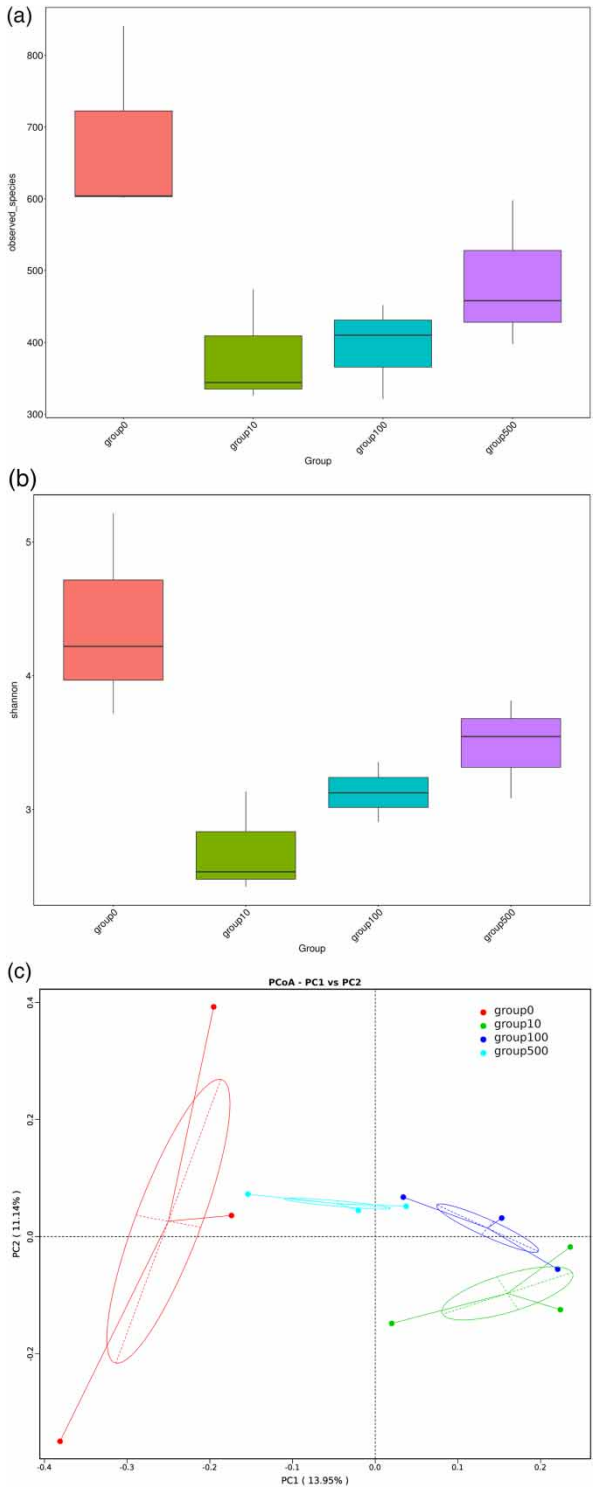

Figure 1 | Effects of DCAcAm on the composition of microbiota in the gut, as detected by 16S rRNA gene sequencing. (a) Observed species and (b) Shannon index of the diversity of gut microbiota after DCAcAm exposure. (c) Gut microbiota patterns in controls and DCAcAm-treated groups differentiated by principal coordinate analysis.

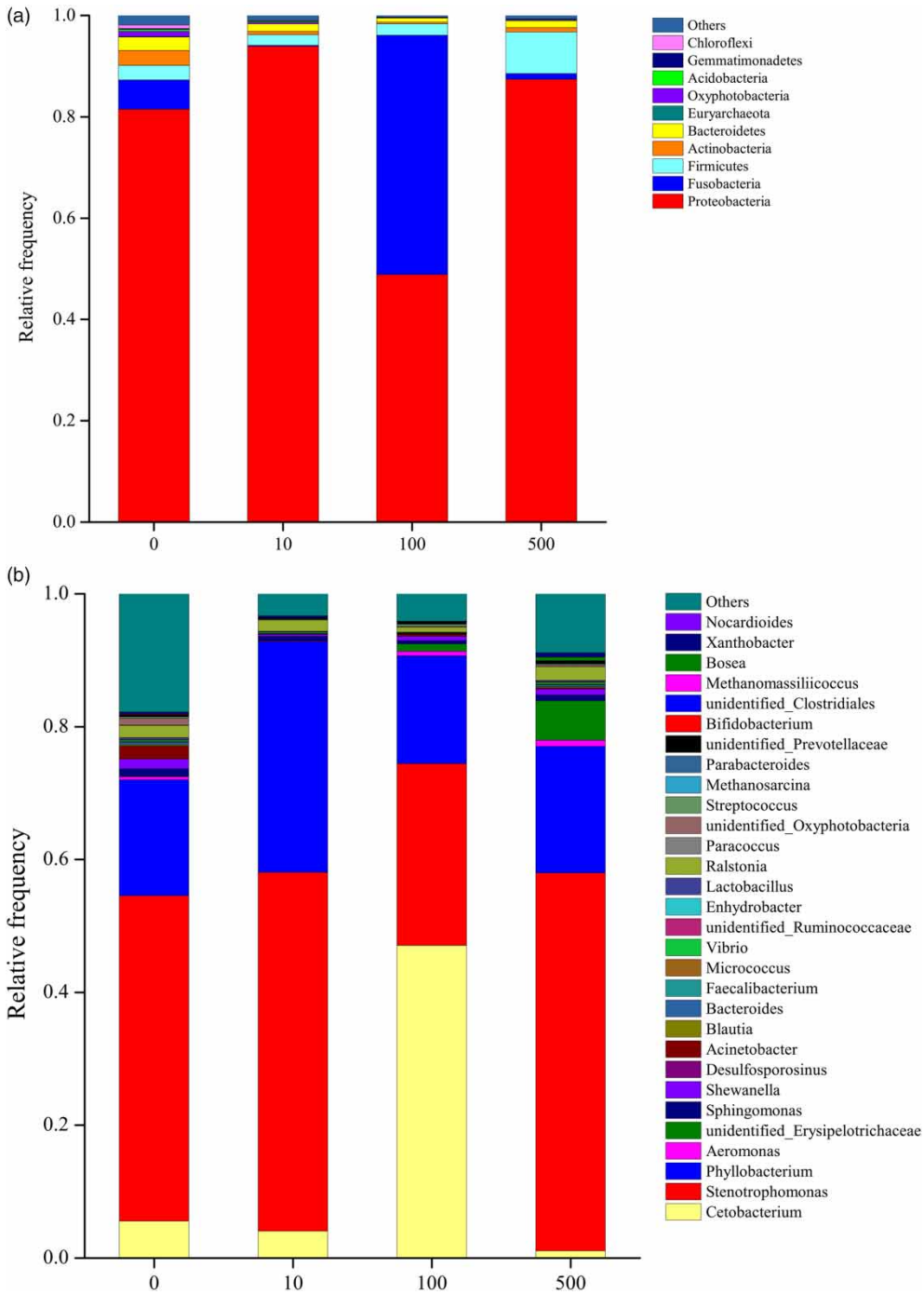

Figure 2 | Relative abundance of: (a) top 10 phyla and (b) top 30 genera after exposure to 0, 10,100 and 500 μg/L DCAcAm, respectively.

Dysbiosis of gut microbiota induced by DCAcAm

Presented here is the first observation that DCAcAm can induce microbiota dysbiosis in the gut of adult zebrafish. At phylum level, Proteobacteria were dominant in all groups, representing 81.48%, 93.96% and 87.44% in the control, 10 and 500 μg/L DCAcAm-treated groups, respectively (Figure 2(a)). Previous studies using zebrafish also

identify the dominance of gut microbiota by Proteobacteria (Chen *et al.* 2018a; Jin *et al.* 2018), although Davis *et al.* (2016) observed highest abundance of Fusobacteria in zebrafish intestines. These geographical differences in gut microbial community of domesticated zebrafish have historical connections with laboratory aquaculture facilities (Chen *et al.* 2018a). Interestingly, the relative abundance of Proteobacteria was decreased from 81.48% in the control group to 48.89% after 100 μg/L DCAcAm exposure for 30 days (Figure 2(a)). In contrast, the composition of Fusobacteria increased from 5.8% in the control group to 47.22% in the 100 μg/L DCAcAm-treated group. Furthermore, the composition of Firmicutes in the control group was approximately 2.92%, but it increased to approximately 8.21% in the 500 μg/L DCAcAm-treated group. Similar results were obtained in the study of Jin *et al.* (2018) that showed the abundance of Proteobacteria decreased significantly and the abundance of Fusobacteria and Firmicutes increased in the gut of zebrafish after exposure to 1,000 mg/L 0.5 and 50 mm polystyrene microplastic for 14 days.

At the genus level, *Stenotrophomonas* was the most abundant genus in all groups, with the exception of the 100 μg/L DCAcAm-treated group, which was dominated by *Cetobacterium* (Figure 2(b)). The abundance of *Cetobacterium* slightly decreased in 10 and 500 μg/L DCAcAm-treated groups, and significantly increased in 100 μg/L DCAcAm exposure group (Figure 2(b)). This increased abundance of *Cetobacterium* is consistent with a previous study, which showed that fish had higher abundance of *Cetobacterium* after administration with dexamethasone (Qi *et al.* 2019). *Cetobacterium*, with a role in sulfur metabolism, has been shown to produce vitamin B12 with high efficiency and to ferment peptides and carbohydrates (Bridges *et al.* 2018; Chang *et al.* 2019). Therefore, we assume that the changes in composition of the fish gut microbiome may suggest an adaptive response to increase DCAcAm detoxification; however, further research should be targeted toward elucidating the underlying mechanisms of these perturbations. Furthermore, although the functions of some of the altered bacteria (Figure 2(b)), such as *Phyllobacterium* and *Acinetobacter*, remain unclear in fish, the functions of several other bacteria, including *Stenotrophomonas*, *Bacteroides* and *Ralstonia*, are considered pathogens or opportunistic pathogens in fish and closely related to fish metabolism, disease and inflammation (Smith *et al.* 2011; Janda *et al.* 2016; Jin *et al.* 2018). In addition, the *Aeromonas*, present only in the 100 and 500 μg/L DCAcAm-treated group, are disease-related bacteria and their reproduction has been demonstrated to cause inflammation of the intestinal tract and infection of soft tissue in a previous study (Chen *et al.* 2018b).

CONCLUSIONS

In conclusion, we have observed that different concentrations of DCAcAm can induce microbiota dysbiosis in the adult zebrafish gut after a 30-day exposure. The results may extend our knowledge regarding the correlations of gut microbial community shift with DCAcAm exposure and provide new information concerning DBPs-induced toxicity in zebrafish. Clearly, the dysbiosis of gut microbiota by chronic DCAcAm exposure in this study is only preliminary. Further research should be targeted toward exploring the relationship between gut microbes and host health, and elucidating the underlying mechanisms of these perturbations.

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