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Risks of antibiotic resistance genes and antimicrobial resistance under chlorination disinfection with public health concerns

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ABSTRACT

As a widely used disinfection technology, the effects of chlorination on antibiotic resistome and bacterial community received great scientific concerns, while the pathogens associated health risks kept largely unknown. With this concern, the present study used metagenomic analysis combined with culture method to reveal chlorination effects on antibiotic resistance genes (ARGs) and their bacterial hosts (total microbes and *Escherichia coli*) through simulating the chlorination dosage with human health concerns (drinking water and swimming pool). The resistome profiling showed that chlorination process could significantly decrease both abundance and diversity of total ARGs, while with limited removal rates of 6.0–8.7% for opportunistic pathogens *E. coli* isolates. Of all the observed 515 ARG subtypes, 105 core subtypes were identified and persistent during chlorination for both total microbes and *E. coli*. Antibiotic susceptibility test showed that chlorination treatment could efficiently remove multi-resistant *E. coli* isolates but select for tetracycline resistant isolates. Five ARG-carrying genomes (assigned to Bacteroidetes, Firmicutes, Actinobacteria) enriched by 18.1–102% after chlorination were retrieved by using metagenomic binning strategies. Bray-Curtis dissimilarity, network and procrustes analyses all indicated the remained antibiotic resistome and bacterial community were mainly chlorination-driven. Furthermore, a systematic pipeline for monitoring chlorination-associated antimicrobial resistance risks was proposed. These together enhance our knowledge of chlorination treatment associated public concerns, as important reference and guidance for surveillance and control of antibiotic resistance.

1. Introduction

The emergence, proliferation and persistence of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment due to the intense use of antibiotics in human and veterinary medicine as well as food production is a global public health issue (Pruden et al., 2006; Zhu et al., 2017). ARGs and ARB have been detected in diverse environments, especially aquatic ecosystems are considered to be with higher mobility of organisms and genetic elements (Zhang et al., 2009). Considering the environmental or public health risks induced by microorganisms, chlorination disinfection has been widely applied in water treatments or sanitation (e.g. drinking water (Jia et al., 2020), municipal wastewater (Guo et al., 2015), industrial wastewater (Shin et al., 2017) and swimming pool (Tsamba et al., 2020) as the last and most solid barrier that prevents potential pathogens into the natural environments or human bodies. It has been demonstrated that chlorination disinfection can effectively reduce the absolute abundance (copy/mL) of ARGs (Chao et al., 2013), but may contribute to the enrichment of their relative abundances (copy/cell) (Jia et al., 2020), likely induced by the underlying mechanisms of cross- or co-resistance to disinfectants and antibiotics (Xi et al., 2009). This could be further aggravated by horizontal gene transfer (HGT) of ARGs via mobile genetic elements (MGEs, e.g. plasmids, transposons and integrons) (Jin et al., 2020). Recently, Zhang et al. discovered that chlorine disinfection could increase cell membrane permeability and thus facilitates HGT of ARGs (Zhang et al., 2021). Thus, the survival of microorganisms, especially human pathogens and ARB, may cause great threats to human health and environmental safety (Lin et al., 2017; Ma et al., 2017).

The antimicrobial resistance (AMR) determinants in drinking water may disseminate to human beings via drinking, bathing, and food washed by running water (Ma et al., 2017), and swimming pool is a public site for recreation and sports, referring potential contact and exchange of microorganisms among persons via eyes, skin, nasal cavity,

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oral cavity and urine (Tsamba et al., 2020). Lutz et al., reported 96% of *Pseudomonas aeruginosa* isolates tested from swimming pools and hot tubs of central Ohio area in the United States were multidrug resistant (Lutz and Lee, 2011). Our previous study of surveying antibiotic resistome of tap water samples from 25 cities of 7 countries/regions observed a total of 181 ARG subtypes belonging to 16 ARG types (Ma et al., 2017). Additionally, ARGs of *sul*1, *sul*2, *tet*A and *tet*C were found persistent from source to tap water in the Yangtze River Delta (Yang et al., 2020). Those ARGs and ARB escaping from chlorination disinfection and remained in swimming pool and tap water with high frequency of human contact lead to a possible threat to human health.

Many studies had been conducted on chlorination associated AMR concerns mainly referring the removal rates of drinking water and municipal wastewater treatment process (Chao et al., 2013; Guo et al., 2015; Jia et al., 2019), cultivable isolates survived in drinking water (Bergeron et al., 2015) and swimming pool (Wei et al., 2018), and further focused on viable but non-culturable strains (Lin et al., 2017) and HGT frequency (Zhang et al., 2021) under exposure to chlorination, which promoted knowledge on chlorination associated AMR risks. While a systematic knowledge of AMR risks of total microbes and concerned pathogenic strains during chlorination for public health related water supply—drinking water and swimming pool—is still deficient for oriented monitoring and control. This was largely restricted by the limited live bacterial cells and cultivable isolates remained after chlorination, as an obstacle for observation and further statistical analysis. *Escherichia coli* is opportunistic pathogens, the most widely-used indicator for fecal bacterial contamination according to water quality standards (WHO, 2017), had been reported to carry diverse ARGs (Mario, 2020). Consequently, whether *E. coli* strains may escape from chlorination treatment and be resistant to antibiotics or carry ARGs is key to the expansion of our knowledge on chlorination associated AMR risks.

With the great public concerns on chlorination disinfection effect on ARGs and ARB in human related water environments, we simulated the chlorination conditions of drinking water treatment and swimming pool disinfection and applied metagenomic analysis combined with culture method to 1) investigate the shifts of ARGs carried by total microbes and retrieve ARG-carrying genomes under chlorination, 2) reveal the ARG and ARB shifts of opportunistic pathogen *E. coli* which is indicator of water quality, and further 3) identify the persistent and enriched ARGs and bacterial hosts associated health risks. The flowchart of this study is shown in Supplementary materials Fig. S1. These together are to fill in the research gaps and as reference & guidance for developing risks management strategies and solutions for public health decision-making to enhance drinking water and swimming pool safety.

2. Material and methods

2.1. Batch chlorination experiments

With public health concerns, the exposure concentrations of free chlorine we used are relevant to disinfectant dosages in swimming pool disinfection (2 mg/L) (Qin et al., 2015) and drinking water treatment (4 mg/L) (Zhang et al., 2021), to investigate the impacts of chlorination on ARG and ARB and associated risks. Influent microbes for simulation of chlorination disinfection were collected from Shek Wu Hui wastewater treatment plant (230,000 m^3/d sewage) in Hong Kong. Water samples were stored at 4 °C upon arrival and used within 3 hrs. The stock solution of free chlorine was prepared by using standard iodometric method to dilute ~12% sodium hypochlorite (Fisher Scientific, UK) into 1,000 mg/L chlorine (Albertson, 2007). Free chlorine solution was added to the influent (200 mL/batch, 20 ± 1 °C) to a final chlorine concentration of 2 mg/L and 4 mg/L respectively. The concentration of total and free chlorine was determined using the DPD method (Hach, USA). The chlorination reaction was under agitation at 150 rpm and sampled after 30 min (WHO, 2011). Before sampling, sodium thiosulfate was added to inactivate residual chlorine. To avoid biases, triplicated batch reactors were conducted for both of the two chlorination processes (2 mg/L and 4 mg/L). Three influents not exposed to sodium hypochlorite served as blank samples.

2.2. Total microorganisms and metagenomic sequencing

The total microorganisms in triplicates before and after chlorination (0, 2, 4 mg/L of chlorine) were collected by filtration using a 0.45-μm cellulose ester membrane (Millipore Corp., USA). The membranes were stored at −20 °C before DNA extraction. Genomic DNA was extracted from each sample (membrane) using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA) according to the standard protocol. The DNA concentration was measured using a NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, USA). A 5 μg aliquot of genomic DNA of each sample was used to construct a 350 bp library (Nextera® DNA Library Preparation Kit, Illumina, Inc., USA). Paired-end metagenomic sequencing was performed on an Illumina HiSeq 4000 platform at Beijing Genomics Institute (China). Then the datasets were subjected to quality-filtering, removing the raw reads containing 3 or more ambiguous nucleotides, reads with an average quality score of <20, and artificially identical reads (Yang et al., 2013). After quality control, 4 Gbp metagenomic sequences of each sample were generated with a total of 72 Gb and submitted to the National Center for Biotechnology Information Short Reads Archive database with the accession number of PRJNA755814.

2.3. Isolation of E. coli strains and metagenomic sequencing

Before isolation, the initial concentration of *E. coli* (3.3×10^7 CFU/100 mL) and its removal rates by chlorination disinfection (99.92% for 2 mg/L and 99.9996% for 4 mg/L, respectively) were determined for selection of suitable dilutions. Then, series dilutions of the influents before (10^5–10^6) and after (10^2–10^3 for 2 mg/L, 0–10^1 for 4 mg/L) chlorination disinfection were plated on HiCrome™ *E. coli* Agar B agar medium (Sigma-Aldrich, USA). The blue colonies as *E. coli* isolates appearance were transferred and inoculated into 200 μL Lysogeny Broth (LB) medium in 96-well microplates (Thermo Scientific, USA) and incubated for 24 h at 37 °C. For each isolate, two 10-μL inoculations were transferred into another two 96-well microplates (200 μL LB medium) and incubated for 24 h at 37 °C, preparing for antibiotic susceptibility test (phenotype) and metagenomic analysis of ARGs (genotype), respectively. Triplicates of 95 *E. coli* strains were randomly isolated for each condition of before and after chlorination, and totally 855 *E. coli* isolates were collected for identification of phenotypes and genotypes of antibiotic resistance characteristics of *E. coli* strains. For profiling of ARGs, the equivalent cells of all the 95 strains from each screen were mixed together, and then DNA extraction and metagenomic sequencing were conducted following the methods described in Section 2.2. OD600 was detected using Model 680 Microplate Reader (Bio-Rad, USA).

2.4. Antibiotic susceptibility test

Antibiotic resistance profiles of 855 collected *E. coli* isolates were determined using a 96-well plate-based broth screening assay. Test plates were freshly prepared by to each well adding 200 μL LB medium supplemented with either ampicillin (Amp), chloramphenicol (Chl), tetracycline (Tet), or kanamycin (Kan). One well in each 96-well microplate was bacteria-free as control. The bacteria suspensions were diluted to generate a final bacteria density in the test plates of approximately 5×10^5 CFU/mL. The final antibiotic concentrations in the test plates were 100 μg/mL Amp, 15 μg/mL Chl, 10 μg/mL Tet, and 15 μg/mL Kan, respectively. The plates were incubated at 37 °C and 200 rpm for 20 h. If the $[OD_{600nm}(\text{isolate})\text{-}OD_{600nm}(\text{control})] < 0.03$, the isolate was identified as nongrowth (antibiotic susceptible) (Suzuki et al., 2014;

Wiegand et al., 2008), for identification of either resistant or susceptible isolates.

2.5. Metagenomic analysis and identification of ARGs

All metagenomic sequencing data were searched for ARGs against SARG (v2.0) database using UBLAST strategies with an E-value $\leq 10^{-7}$ (Yin et al., 2018). A sequence was identified as an ARG-like fragment if its best BLASTX alignment to the ARG sequence in the referenced database showed a similarity of ≥80% and alignment length of ≥75% (Yin et al., 2018). The identified ARG-like sequences could be classified into 24 ARG types (e.g., sulfonamide resistance gene) and 1,208 ARG subtypes (e.g., *sul*1, *sul*2, etc.). This annotation pipeline has been validated with a high accuracy of 97–99.5% in previous studies (Yang et al., 2013; Yin et al., 2018). The abundances of ARGs were normalized as the number of copies of ARGs per cell (copy/cell) (Yang et al., 2016), based on the equation in Supplementary materials text S1.

2.6. Statistics analysis

A heatmap plot was conducted using the R program (v3.5.3) to present the abundances of ARGs carried by total microbes and *E. coli*. The Venn diagram showing the number of shared ARG diversity among treatments was generated using the Venn tool termed jvenn (Bardou et al., 2014). ARG profiles of each sample were differentiated by the principal coordinate analysis (PCoA) based on Bray-Curtis distance using R with the Vegan package. Resistome similarity between each treatment was conducted based on Bray-Curtis dissimilarity analysis using R program. Network analysis using the matrix of ARGs (with ARGs-OAP (Yin et al., 2018)) and taxonomic data (with MetaPhlAn2 (Truong et al., 2015)) was performed with R "psychc" and "reshape2" packages, and then visualized using the interactive Gephi platform (v0.9.2). A Procrustes analysis based on the abundance matrix of ARG subtypes and taxonomy was performed using QIIME (Forsberg et al., 2014). Metagenome-assembled genomes (MAGs) was retrieved by using metaWRAP pipeline (v1.2) (Uritskiy et al., 2018) and then compared against SARG (v2.0) (Yin et al., 2018) for identification of ARGs-carrying MAGs (Ma et al., 2016).

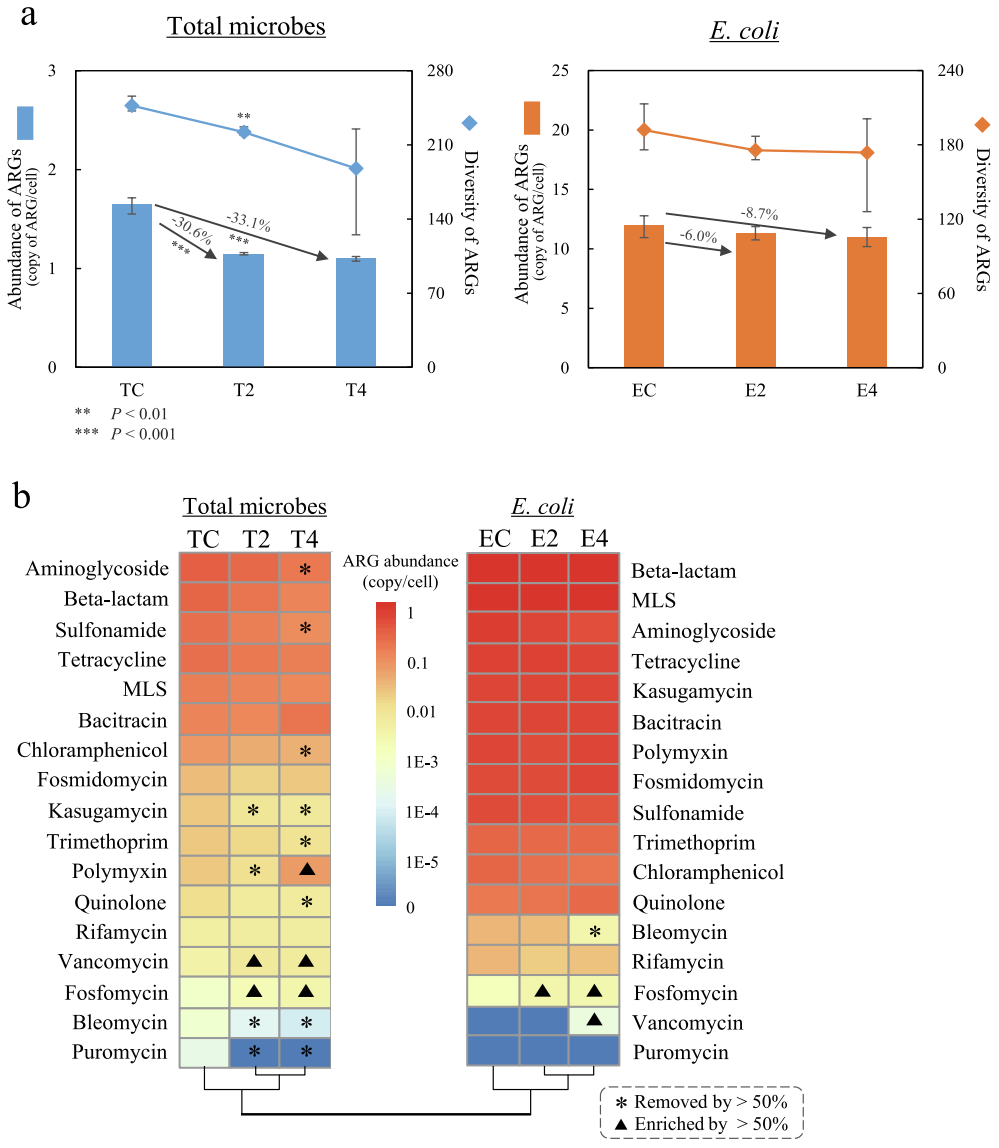

Fig. 1. Shifts of ARGs abundance after chlorination. (a) Shifts of ARGs abundance carried by total microbes and *E. coli*, respectively. (b) Abundance shifts of ARG types. For sample ID "TC, T2, T4, EC, E2 and E4", 'T' indicates total microbes, 'C' indicates control sample before chlorination, '2' presents exposure to 2 mg/L of chlorine, '4' presents exposure to 4 mg/L of chlorine, and 'E' indicates *E. coli*.

3. Results

3.1. Chlorination disinfection decreased ARG abundances

Here investigated comprehensive profiles of ARGs and their shifts during chlorination. For initial influent sample, we identified abundance of averagely 1.65 and 12.0 copies/cell carried by total microbes and *E. coli* isolates, respectively. Chlorination disinfection then caused a significant decrease of ARG abundance ($p < 0.001$) carried by total microbes, with removal rates of 30.6% (2 mg/L, $P < 0.001$) and 33.1% (4 mg/L, $P < 0.001$) (Fig. 1**a**). As opportunistic pathogens, *E. coli* isolates had a slight decrease of ARG abundance after chlorination, with removal rates of 6.0% (2 mg/L) and 8.7% (4 mg/L). Thus, *E. coli* was in significantly higher ARG abundance than total microbes ($P < 0.001$), while ARGs carried by total microbes are more sensitive under chlorination disinfection. Moreover, comparing with 2 mg/L of free chlorine, the chlorination under 4 mg/L of free chlorine had a relatively higher removal rates of ARGs by 2.5% and 2.7% for total microbes and *E. coli* isolates, respectively.

A total of 17 ARG types were detected in total microbes, of which puromycin resistance genes were not observed carried by *E. coli*. Chlorination disinfection significantly removed 8 ARG types (aminoglycoside, sulfonamide, chloramphenicol, kasugamycin, trimethoprim, quinolone, bleomycin, and puromycin) for total microbes (by > 50%), but only highly removed one ARG type for *E. coli* (bleomycin-ARGs) by 91.2% under exposure to 4 mg/L of chlorine (Fig. 1**b**). Fosfomycin resistance genes were enriched by >50% observed in both total microbes and *E. coli* after chlorination disinfection. Although abundances of most detected ARG types were decreased after chlorination, with 76% of ARG types for total microbes and 56% for *E. coli*, several ARG types were observed significantly removed under low-dose chlorination but showed enrichment under high-dose chlorination, especially for total microbes, such as bacitracin- (10.7% removed under 2 mg/L, 54.9% enriched under 4 mg/L) and polymyxin-ARGs (51.5% removed under 2 mg/L, 216% enriched under 4 mg/L).

To further explore the chlorination effects on ARG shifts, the abundances of ARG subtypes with initial abundances higher than 0.1 copy/cell were present in Fig. 2. Among the 37 dominant ARG subtypes carried by total microbes, 5 subtypes were significantly removed by 2 mg/L chlorination, and 16 subtypes were significantly removed by 4 mg/L chlorination (Fig. 2**a**). For the 52 dominant ARG subtypes that carried by *E. coli*, 3 subtypes (*aph*(3′)-I, CTX-M, and OXA-10) were significantly removed by exposure to 2 mg/L of chlorine, and 6 subtypes (*aac*(3)-II, *aph*(3′)-I, *aph*(3′′)-I, CTX-M, OXA-10 and *tet*M) were significantly removed at dosage of 4 mg/L of chlorine (Fig. 2**b**). In contrast, specific ARGs were enriched during chlorination that *bac*A and *arn*A in total microbes were significantly enriched after chlorination with 4 mg/L of chlorine, while *qnr*B and *tet*D carried by *E. coli* were significantly enriched after both of the chlorination treatments.

3.2. Shifts of ARGs diversity and identification of persistent ARGs

Number of ARG subtypes was used to assess ARG diversity carried by microorganisms (Yin et al., 2018). In the present study, there were

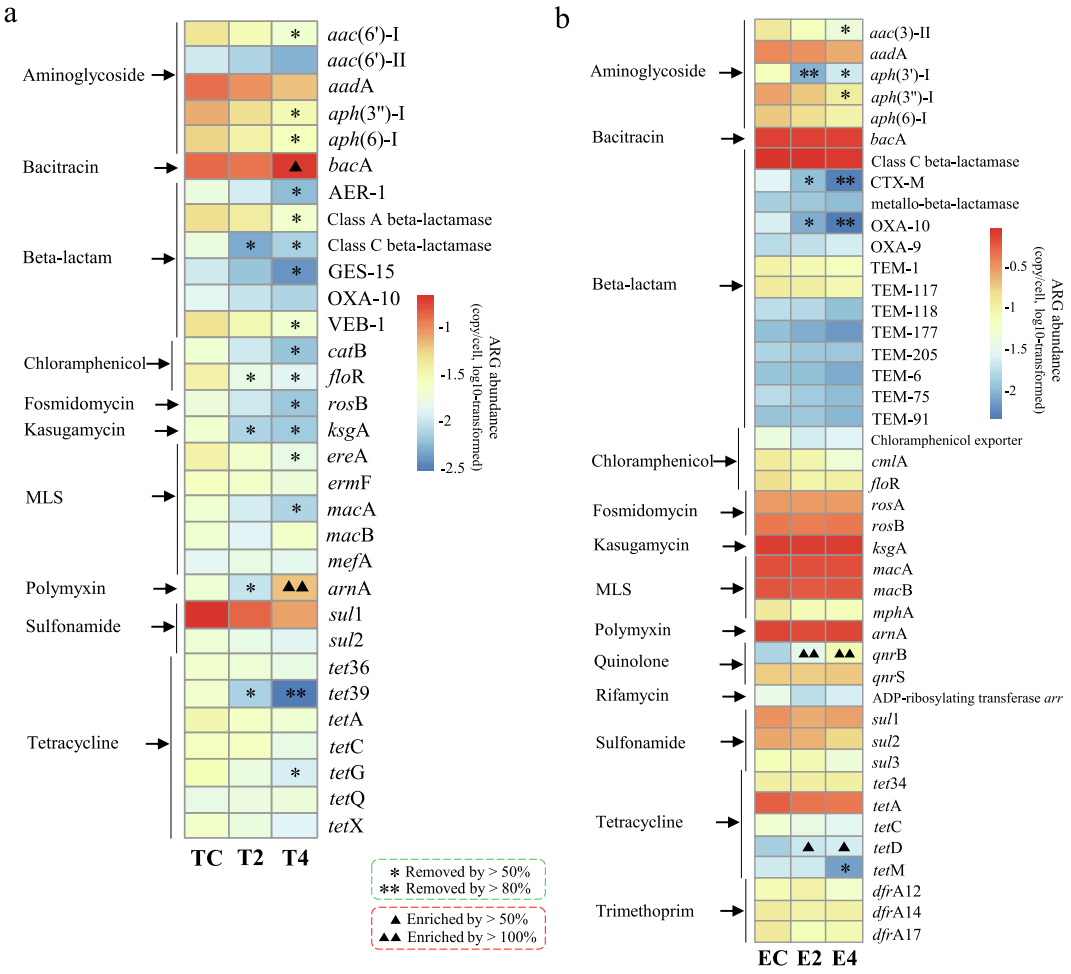

Fig. 2. Abundance shifts of dominant ARG subtypes (with initial abundance of > 0.1 copy/cell) after chlorination. (a) Shifts of dominant ARG subtypes carried by total microbes. (b) Shifts of dominant ARG subtypes carried by *E. coli*.

totally detected 515 ARG subtypes. Before chlorination, there observed averagely 247 and 192 subtypes carried by total microbes and *E. coli*, respectively (Fig. 1**a**). Similar to ARG abundance, chlorination disinfection also contributed a decreasing trend of average diversity index for total microbes and *E. coli*. Under the pressure of chlorination treatment, there remained 224 and 185 ARG subtypes carried by total microbes and *E. coli* respectively, identified as persistent ARGs (Fig. 3**a**). Among them, 105 ARG subtypes were persistent in both total microbes and *E. coli*, identified as core ARGs (Fig. 3**b**, **Table S2**), encoding resistance to a total of 15 antibiotic types, with 38% attributed to beta-lactam-ARGs, followed by 15% (tetracycline-ARGs), 7.6% (aminoglycoside-ARGs) and 7.6% (MLS-ARGs). Bacitracin resistance gene *bac*A was in relatively high abundance in both of total microbes and *E. coli*, and kept stable during chlorination (Fig. 3**c**), while top persistent ARGs of *bla*VEB-1, *aac*(6′)-I, cAMP-regulatory protein and *ere*A carried by *E. coli* slightly increased. Thus, with the public concerns, these revealed persistent and core ARGs should be paid more attentions on their removal fates and associated potential health risks.

3.3. Resistome shifts of total microbes and E. coli based on similarity analysis

Although levels of abundance and diversity showed significant shifts during chlorination, it is still difficult to clarify the differentiation degree only based on the observations of total abundance, or specific ARG type/subtypes. Thus, principal co-ordinates analysis (PCoA) was used to visualize the shifts of antibiotic resistome, to compare total microbes *vs* *E. coli*, and under 2 mg/L *vs* 4 mg/L of chlorine. PCoA plot in Supplementary materials Fig. S4 showed that the antibiotic resistome carried by total microbes significantly changed, with a higher discrepancy after 4 mg/L chlorination comparing with 2 mg/L chlorination disinfection. In contrast, no significant discrepancy was observed for antibiotic resistome of *E. coli* isolates. Further quantification indicated that chlorination disinfection differs resistome similarity of total microbes to be <0.8, while >0.92 for *E. coli* isolates, indicating the escaped *E. coli* strains in chlorinated water supply may pose persistent AMR risks. The similarity of resistome with 4 mg/L chlorination was lower than that of 2 mg/L chlorination for both of the total microbes and *E. coli* isolates, indicating a higher sensitivity under the high chlorination pressure.

3.4. Shifts of antibiotic resistant E. coli isolates after chlorination

To reveal the impacts of chlorination treatment on antibiotic susceptibility of strains, antibiotics of Amp, Tet, Kan and Chl were used for antibiotic susceptibility test, to assess the antibiotic resistant *E. coli* isolates before and after chlorination. A total of 855 *E. coli* bacterial strains were isolated and subjected to antibiotic resistance profiling. Overall, 475 isolates were observed to be resistant to at least one of the tested antibiotics, with averagely 55.6%, 50.0% and 59.4% for before and after 2 and 4 mg/L treatments, respectively. After 2 mg/L chlorination, there observed significantly lower proportions of *E. coli* isolates resistant to Amp, Tet, Kan and Chl, decreased by averagely 21%, 6%,

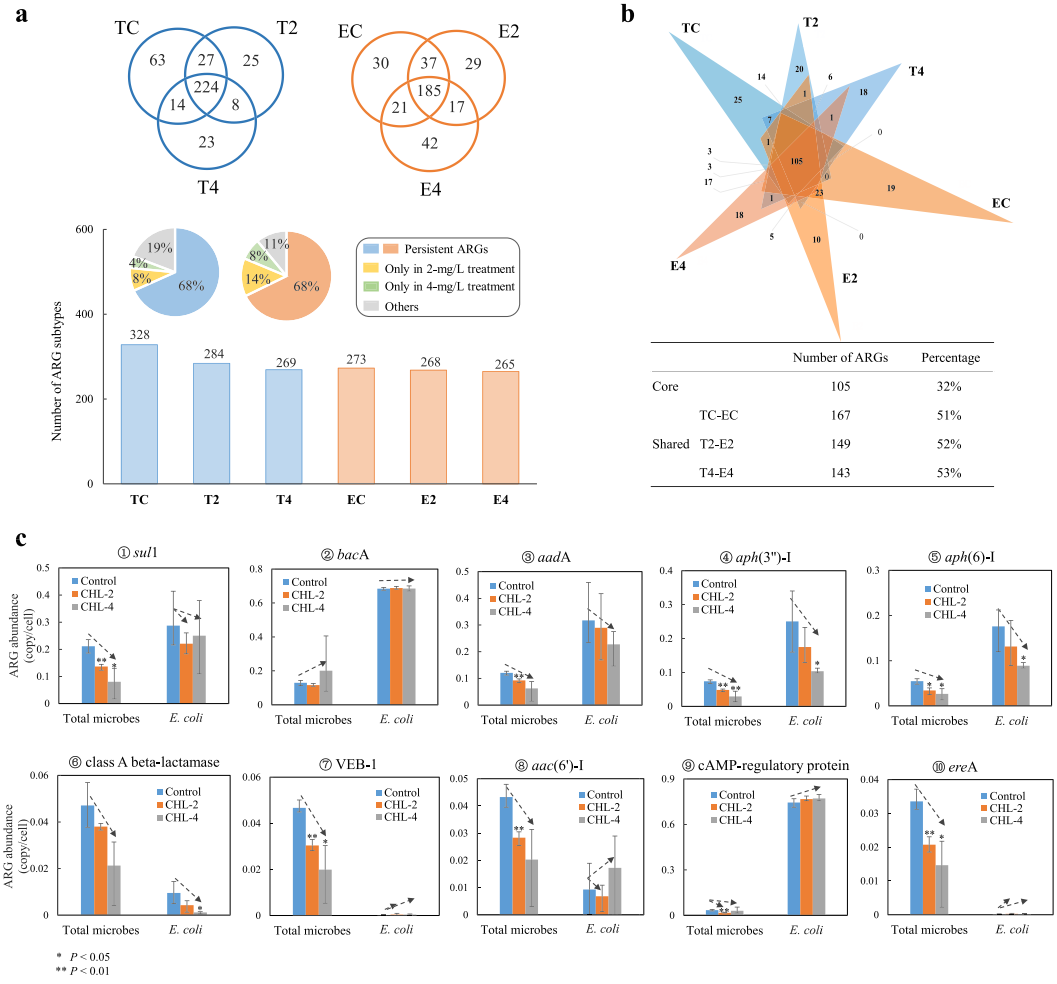

Fig. 3. Shifts of ARGs diversity after chlorination. (a) ARG diversity (number of subtypes) before and after disinfection of 2 and 4 mg/L chlorine, carried by total microbes and *E. coli*, respectively. (b) Core and shared patterns of ARG diversity before and after chlorination treatment. (c) The occurrence and shifts of top 10 persistent and core ARGs. CHL-2: treatment with 2 mg/L of chlorine; CHL-4: treatment with 4 mg/L of chlorine.

83% and 20%, respectively (Fig. 4**a**). While after 4 mg/L chlorination, there observed lower proportions of isolates resistant to Amp, Kan and Chl, but an increase for percentage of tetracycline-resistant *E. coli* isolates by averagely 24.4%.

Multi-resistant isolates (resistant to ≥2 tested antibiotics) resistant to Amp-Tet, Tet-Chl and Amp-Tet-Chl were persistent after chlorination, while isolates resistant to Tet-Kan and Amp-Kan-Chl were removed after chlorination (Fig. 4**b**). *E. coli* isolates resistant to Amp-Kan-Chl were not observed in both of the before and after chlorination treatments. Among all the isolates, averagely 38.6%, 26.4% and 32.6% were multi-resistant before and after 2 and 4 mg/L treatments, respectively (Fig. 4**c, d**). Under further observation, the percentage of sensitive isolates did not significantly change, but multi-resistant isolates were decreased by 31.6% and 15.5% for 2 and 4 mg/L chlorination, respectively (Fig. 4**c, d**). Thus, chlorination disinfection may not efficiently decrease the proportions of antibiotic resistant *E. coli*, but could remove multi-resistant strains to Amp, Chl, Kan and Tet.

3.5. Antibiotic resistome after chlorination driven by bacterial communities

A total of 12 bacterial phyla and 113 genera were detected before and after chlorination by profiling of total microbes. As shown in Fig. 5**a**, the relative abundance of Proteobacteria significantly decreased by 17.1% and 9.8% respectively, while the relative abundances of Actinobacteria, Firmicutes, and Thermotogae averagely increased after chlorination. At genus level, *Escherichia*, *Acinetobacter* and *Aeromonas* were significantly decreased by chlorination, especially by >70% after 4 mg/L treatment. In contrast, chlorination could increase the relative abundances of genera *Pseudomonas* and *Bacteroides*. Similarly to the shifts of resistome composition, the bacterial communities were differed more significantly by 4 mg/L chlorination in community similarity of 0.71 with control, indicated by Bray-Curtis dissimilarity analysis (Fig. 5**b**). The observed consistent trend of resistome and communities shifts indicated that the changes of ARGs could be mainly driven by bacterial communities under chlorination treatment. Thus, network and procrustes analysis based on the matrix of ARGs and taxonomy were further used for illustrating their correlations.

A total of 34 bacterial genera and 51 ARG subtypes constituted the network plot under cutoffs of $r > 0.9$ and $P < 0.01$ (Fig. 5**c**). Among these potential bacterial hosts of ARGs, *Aeromonas* was found highly correlated with diverse ARGs, including *aph*(3'')-I, *bla*AER-1, class A beta-lactamase, *bla*GES-15, *bla*OXA-13, *bla*OXA-21, *ros*B, *sul*1, *tet*E, *tet*G, and *dfr*A14. Followed by *Acinetobacter* that was correlated with 6 ARGs, including *aad*A, *bla*GES-15, *bla*OXA-147, *bla*VEB-1, *ros*B and *dfr*A14. Consistently, these correlated ARGs were highly decreased, matching with the shifts of the dominant hubs *Aeromonas* and *Acinetobacter*. While *Bacteroides* with averagely 26.6% enriched by chlorination was significantly correlated with chloramphenicol resistance gene *cat*Q whose abundances increased by averagely 38.7%. To further clarify the shift patterns of ARGs carried by different bacterial communities under chlorination, a Procrustes analysis based on a one-way ANOVA test with Tukey post-hoc tests was performed (Fig. 5**c**). The results ($M^2 = 0.145$) showed these samples could be distributed separately, indicating the resistome was highly driven by bacterial communities under different chlorination treatments.

3.6. Enriched ARG-carrying bacterial genomes by chlorination

To further reveal the host-associated health risks of resistome during chlorination disinfection, metagenomic binning strategy was performed for retrieve of ARG-carrying genomes (ACGs). A total of 19 ARG-carrying draft genomes were recovered from metagenomes (Fig. 6**a**), and 5 of them were found enriched by chlorination (Fig. 6**b, c**). Among them, bin3 (assigned to phylum Bacteroidetes, carrying OXA-10, *cml*A, *ere*B and *tet*W) in the highest original abundance (8.8 ×/Gb on average) among enriched ACGs increased by 19.6% and 18.8% under 2 and 4 mg/L chlorination respectively. The highest enriched was bin14 (assigned to

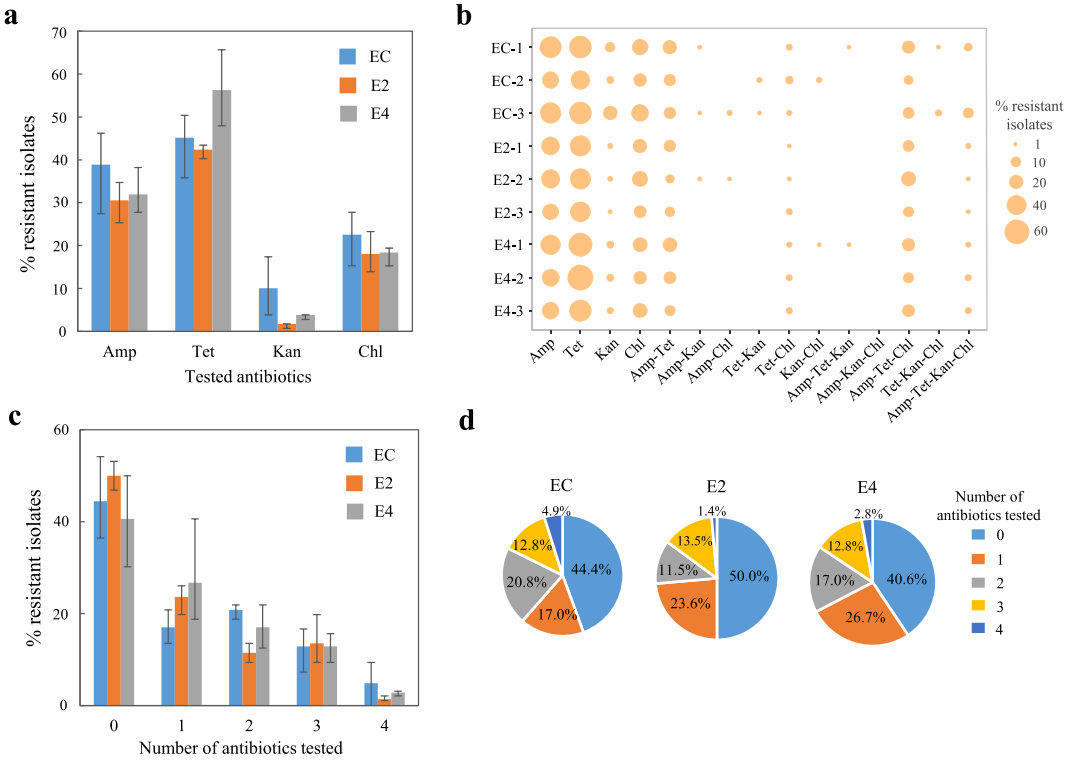

Fig. 4. Shifts of antibiotic resistant *E. coli* isolates after chlorination. (a) Percentage shifts of antibiotic resistant isolates after chlorination. (b) Multi-resistance of *E. coli* strains before and after chlorination treatment. (c) Percentages of susceptible and multi-resistant isolates. (d) Proportions of susceptible and multi-resistant isolates.

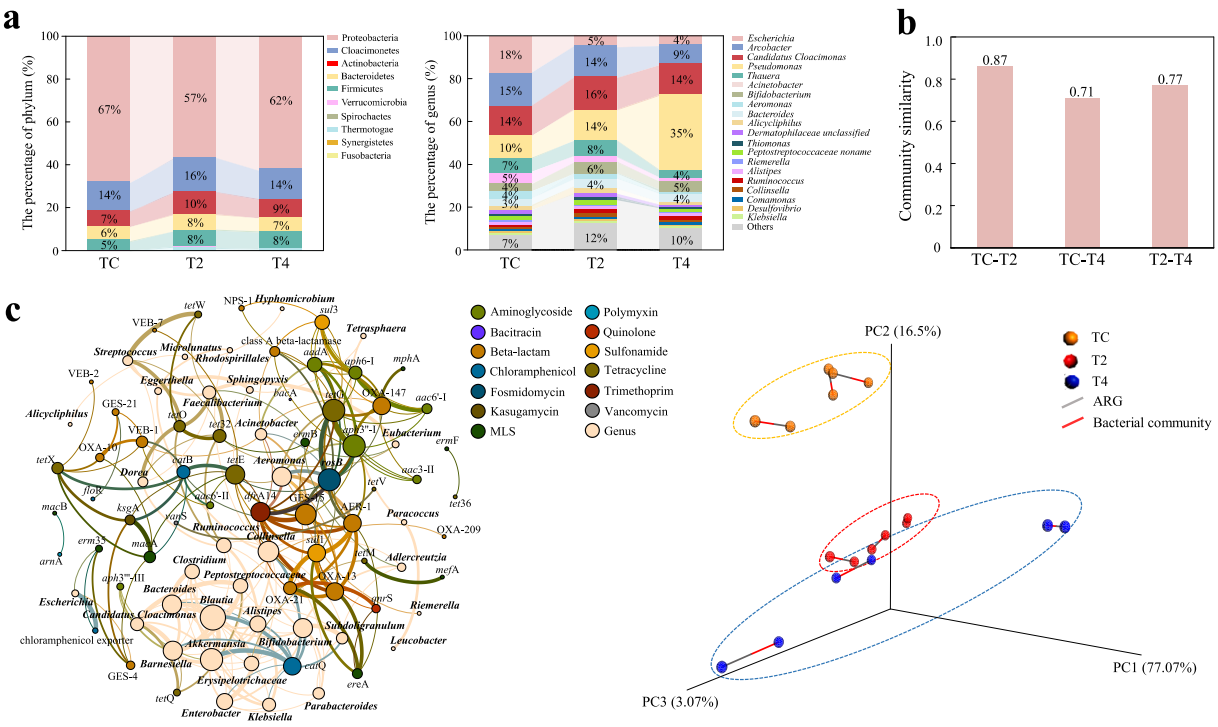

Fig. 5. Shifts of bacterial communities after chlorination disinfection. (a) The shifts of bacterial phyla and top 20 bacterial genera. (b) Comparison of community similarity based on Bray-Curtis dissimilarity analysis based on taxonomic data in genus level. (c) Network and Procrustes analysis based on matrix of ARG abundance and bacterial communities. The size of each node of network is proportional to its number of connections, and the edges present the correlation between two nodes. A connection represents a strong (Spearman's rank correlation coefficient $r > 0.9$) and significant (P value < 0.01) correlation. Procrustes analysis depicts correlation between ARG content (Bray-Curtis) and bacterial community (Bray-Curtis), $M^2 = 0.145$.

family Nocardioidacae, carrying *arr*) whose abundance increased by 102% under 2 mg/L chlorination, followed by bin7 (assigned to phylum Firmicutes, carrying *tet*32 and *vat*B) with highly enriched by 55.3% under 4 mg/L chlorination. This indicated that chlorination could efficiently remove ARG-carrying genomes, but specific chlorination resistant bacteria that harbored diverse ARGs can escape and be relatively enriched, posing potential health risks to human beings.

(Since the embedded Fig. 6 is not clear enough, the separate file of Fig. 6 in high solution has been uploaded with the manuscript file for review. Please see the high-solution Fig. 6 after "references section" in this merged PDF.)

4. Discussion

The supplies of sanitary swimming pool and safe drinking water are mainly based on chlorination treatment to disinfect microorganisms. Herein whether the chlorination process could efficiently remove ARGs and ARB, and what kinds remained with potential health risks of great public concerns. The present study used metagenomic technique combined with traditional culture based methods to reveal the selection of chlorination treatment on antibiotic resistome carried by microbiome and opportunistic pathogens.

4.1. Underestimated risks of antibiotic resistome carried by pathogens

Here, public health concerned chlorination disinfection can significantly remove ARGs carried by total microbes by 30.6–33.1% ($P < 0.01$), but limitedly remove antibiotic resistome of *E. coli* isolates by 6.0–8.7%. Similarly, previous studies also observed a significant decrease of total ARGs after chlorination treatment (Jia et al., 2020; Lin et al., 2016), but the associated health risks may be underestimated without considering the pathogenic indicators. The relatively high ARG abundance of *E. coli* and their high resistome similarity after chlorination observed in this study further indicated the escaped *E. coli* strains in chlorinated water supply pose persistent high AMR risks. Based on the traditional culturing methods, Bai et al., identified the enhanced antibiotic resistance of culturable bacteria after drinking water treatment (Bai et al., 2015), while *E. coli* is rarely specifically isolated and monitored after chlorination except for under low level chlorination (e.g., 0.5 mg Cl_2/L (Lin et al., 2017)) because of the extremely low biomass after chlorination making it difficult to isolate strains with a sufficient quantity for statistics. In addition, their focuses on specific antibiotic resistance or ARGs greatly hindered a comprehensive knowledge of chlorination-associated health risks of antimicrobial pathogens. The underestimation of resistance risks of *E. coli* could be further aggravated by the chlorination-induced enrichment of viable but non-culturable *E. coli* (Lin et al., 2017). Therefore, antibiotic resistant opportunistic pathogens associated health risks should be paid more attentions for emphatically monitoring and control.

4.2. Chlorination selectively enrich specific ARGs and select resistance phenotype

Although chlorination disinfection could efficiently decrease the total abundance of ARGs carried by total microbes and have slight removal effect on that carried by *E. coli*, it could selectively enrich specific ARGs, such as, *bac*A and *arn*A of total microbes, *qnr*B and *tet*D of *E. coli*. Similarly, Jia et al., reported chlorine disinfection enriched specific ARGs in drinking water, especially *bac*A encoding resistance to bacitracin (Jia et al., 2015). With the enrichment of *tet*D carried by *E. coli*, the significant enrichment of tetracycline resistant isolates after chlorination of 4 mg Cl_2/L was discovered though the proportions of multi-resistant isolates significantly decreased. Jahantigh et al., had reported the presence of *tet*D and antibiotic sensitivity to tetracycline had a significant relationship in *E. coli* strains isolated from colibacillosis infections (Jahantigh et al., 2020). Moreover, higher frequency of

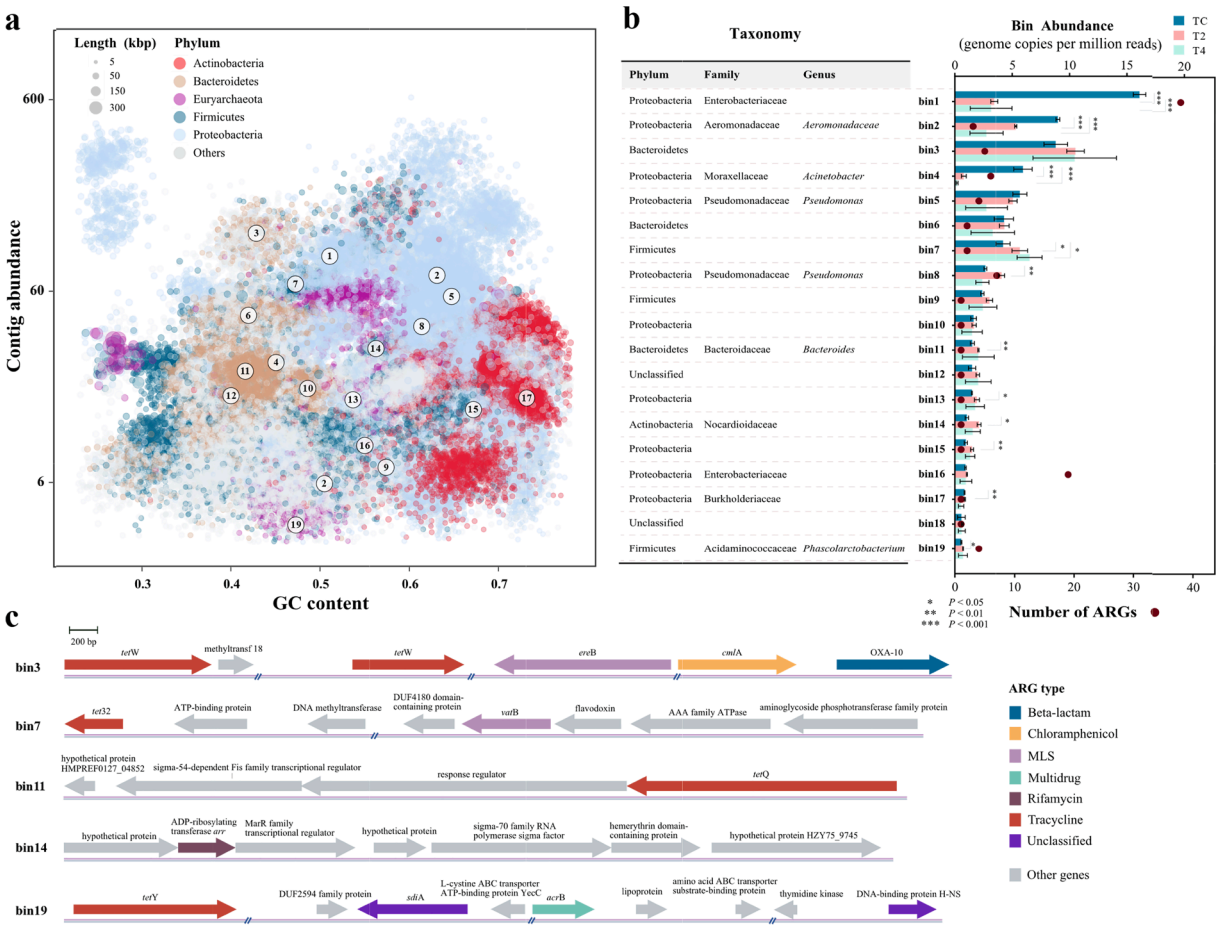

Fig. 6. Retrieved ARG-carrying genomes from metagenomes. (a) Recovered ARG-carrying draft genomes using metagenomic binning. Circles represent scaffolds, scaled by the square root of their length and colored according to phylum-level taxonomic affiliation. Extracted ARG-carrying draft genomes are numbered. (b) Abundance and taxonomy of recovered 19 ARG-carrying genomes, and number of ARGs they carried. (c) The resistome of ARG-carrying genomes which were enriched by chlorination.

tetracycline resistant *E. coli* was previously reported in chlorinated drinking water, reclaimed water, and greywater (Huang et al., 2013; Troiano et al., 2018). These together indicated tetracycline resistant *E. coli* had a potentially higher tolerance to chlorination, and *tet*D could be the ARG contributor. Thus, chlorination process could selectively enriched specific ARGs, and correspondingly drive the resistance phenotypes, with public health concerns.

4.3. Persistent ARGs and their bacterial hosts

The chlorination process decreased ARG diversity for both total microbes and *E. coli*, while 224 and 185 ARG subtypes respectively of them were found persistent during chlorination. Although chlorination could decrease total abundances of persistent ARGs by 59.8% and 47.6% for total microbes and *E. coli* respectively, the ones escaped from chlorination and remained in drinking water and swimming pool may pose potential risks to human health, via carried by pathogens or horizontal gene transfer by MGEs. Of which, the dominant persistent ARGs with enriched abundance deserve more attentions, e.g., *bac*A, VEB-1, *aac*(6′)-I. *Bac*A gene encoding bacitracin resistance had been frequently reported to be dominant persistent and enriched during drinking water treatment processes (Jia et al., 2020). Chlorination can kill bacteria by destroying cell wall, but *bac*A is essential for the biosynthesis of peptidoglycan and other cell wall components (Ghachi et al., 2005), which may contribute to the survival of their bacterial hosts under chlorine stress.

Furthermore, here Bray-Curtis dissimilarity together with network and procrustes analyses all indicated the profiles of antibiotic resistome remained after chlorination were mainly driven by shifts of bacterial community. Previous studies based on culturing methods and metagenomic analysis both indicated bacterial community shift caused by disinfection is the major driver shaping the antibiotic resistome (Jia et al., 2020; Shi et al., 2013), and the difference in chlorine sensitivity among bacterial populations in drinking water may be the main reason for the succession dynamics and diversification of microbial community (Jia et al., 2015). Thus, the potential bacterial hosts of ARGs indicated by network analysis and recovered ARG-carrying MAGs by metagenomic binning strategies, especially for those persistent and enriched ARGs, provided us the reference and guidance of ARG removal during chlorination for public water use.

4.4. Perspectives

Our study revealed health risks associated from persistent ARGs, enriched ARGs, and bacterial hosts (especially pathogenic indicator and enriched bacteria) during chlorination treatment. The remaining ARGs and ARB may pose threats to human beings via direct contact or intake and even exacerbated through HGT with human pathogens. With this concern, a catalogue of ARGs and the bacteria harboring them escaped from chlorination should be established for further intensive monitoring and control, considering 1) persistent and enriched ARGs, and 2) persistent and enriched bacterial hosts (culturable or assembled genomes), and potential bacterial hosts indicated by correlation-based analysis. The research pipeline and perspectives are proposed in

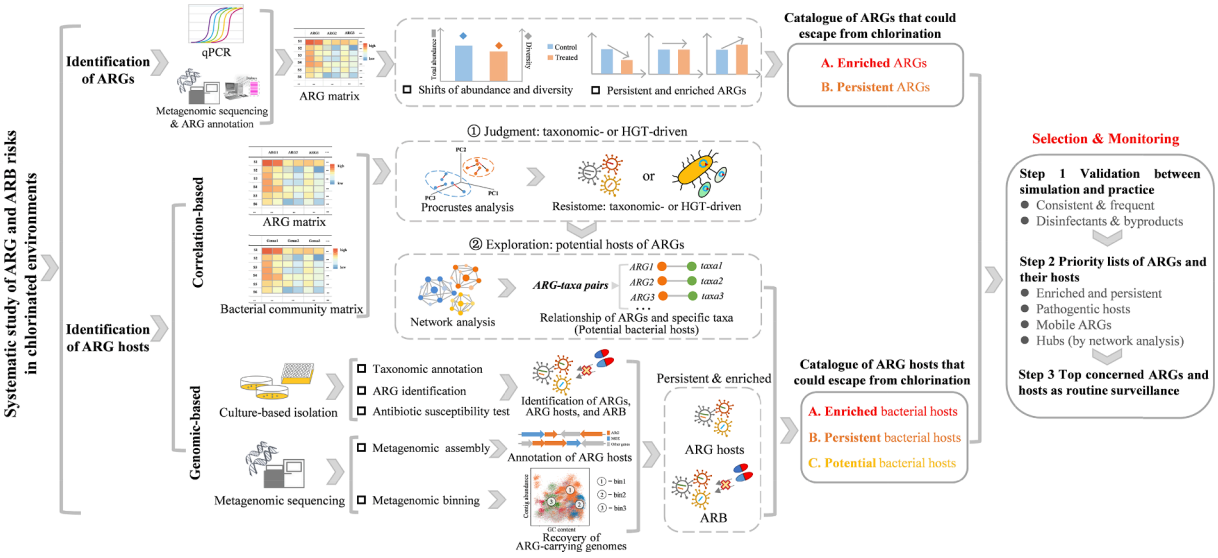

Fig. 7. Proposed systematic analysis of ARG and ARB risks in chlorinated environments and development of priority list for surveillance and control.

Fig. 7. In future, more researches and reviews are warranted for great efforts on collection and summary of ARGs and bacterial hosts escaped from or enriched by chlorination. Although previous researches had obtained rich observations on specific enriched and persistent ARGs (e.g., *amp*C (Shi et al., 2013), *bac*A (Jia et al., 2015), *qnr*A (Wang et al., 2017)), their bacterial hosts and associated contributions remained largely unexploited, or mainly predicted for potential hosts based on correlation analysis. Culture- and genomic-based host identification could provide us further solid evidence for bacterial vectors of ARGs. Till now, several studies had recognized that antibiotic resistome are largely shaped by bacteria community survival from chlorination (Jia et al., 2020; Shi et al., 2013), thus, the foremost for control and alleviation of ARGs is to identify their bacterial hosts (contributors), based on the combination of lab-based simulation and field-based practice samples. And further based on sufficient materials to formulate priority lists of ARGs and their hosts, and develop routine surveillance and oriented control strategies. This will facilitate our evaluation and minimization of health risks associated with ARGs and ARB in chlorinated water systems, for both of the public and ecological health concerns.

5. Conclusion

This study used metagenomic analysis combined with culture method and revealed the chlorination effects on ARGs and ARB with human health concerns through simulating the public health related chlorination dosage. For total microbes and pathogen indictor *E. coli*, chlorination process could decrease both their ARGs abundances, while with limited removal rates of 6.0–8.7% for *E. coli* isolates. Of all the observed 515 ARG subtypes, 105 core subtypes were identified and persistent during chlorination for both total microbes and *E. coli*. ARGs of *bac*A and *arn*A, *qnr*B and *tet*D were found enriched by >50% for total microbes and *E. coli*, respectively. Five ARG-carrying genomes enriched by 18.1–102% after chlorination were retrieved by using metagenomic binning strategies. For phenotype, chlorination treatment could efficiently remove multi-resistant *E. coli* isolates but select for tetracycline resistant isolates under 4 mg/L chlorination, in close relation to their enrichment of *tet*D. Our novel insights into the chlorination treatment for effects assessment of bacterial genotypes and phenotypes comprehensively revealed the health risks within 1) specific ARGs and resistance phenotype selectively enriched by chlorination, 2) underestimated risks of antibiotic resistome carried by opportunistic pathogens, and 3) persistent/enriched ARGs and their bacterial hosts. These together with proposed perspectives requiring more efforts on formulating priority lists of ARGs and their bacterial hosts will enhance our knowledge of chlorination associated public concerns and could propel future monitoring and managements.

CRediT authorship contribution statement

Liping Ma: Conceptualization, Methodology, Data curation, Software, Visualization, Funding acquisition, Writing – original draft, Writing – review & editing. **Huiying Yang:** Data curation, Software, Visualization. **Lei Guan:** Software, Visualization. **Xiaoyu Liu:** Data curation. **Tong Zhang:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106978.

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