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Recovery of microbiological quality of long-term stagnant tap water in university buildings during the COVID-19 pandemic

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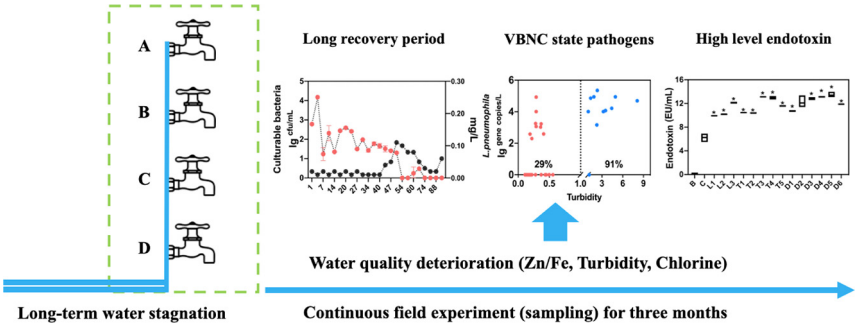
HIGHLIGHTS

- Long-term water stagnation resulted deteriorated water quality.
- Pathogens were found in long-term stagnant water (LTSW) in the form of VBNC.
- *L. pneumophilia* had high frequency detection in LTSW with turbidity > 1 NTU.
- Water quality recovery period of microbial parameter is the longest.
- High level of endotoxin in LTSW was detected.

GRAPHICAL ABSTRACT



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ABSTRACT

Stagnant water can cause water quality deterioration and, in particular, microbiological contaminations, posing potential health risks to occupants. University buildings were unoccupied with little water usage during the COVID-19 pandemic. It's an opportunity to study microbiological quality of long-term stagnant water (LTSW) in university buildings. The tap water samples were collected for three months from four types of campus buildings to monitor water quality and microbial risks after long-term stagnation. Specifically, the residual chlorine, turbidity, and iron/zinc were disqualified, and the heterotrophic plate counts (HPC) exceeded the Chinese national standard above 100 times. It took 4-54 days for these parameters to recover to the routine levels. Six species of pathogens were detected with high frequency and levels (10^1-10^5 copies/100 mL). Remarkably, *L. pneumophilia* occurred in 91% of samples with turbidity > 1 NTU. The absence of the culturable cells for these bacteria possibly implied their occurrence in a viable but non-culturable (VBNC) status. The bacterial community of the stagnant tap water differed significantly and reached a steady state in more than 50 days. Furthermore, a high concentration of endotoxin (>10 EU/mL) was found in LTSW, which was in accordance with the high proportion of dead bacteria. The results suggested that the increased microbiological risks require more attention and the countermeasures before the building reopens should be taken.

1. Introduction

The stagnation of tap water is usually a problem of individual and occasional cases in residential community buildings. However, it may be recurrent incidents in public buildings like university buildings because of the periodical reduction of water consumption during winter and

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summer vacations, which can last for one to three months. All of these phenomena cannot compare to the situation during the COVID-19 pandemic. Hundreds of millions of university students and faculty members all over the world were absent from campus for several months. For instance, all of the campus in China was unoccupied from December 2019, and the universities did not reopen until May or September 2020. Stagnation has been associated with the deterioration of water quality at the distribution system level with the ubiquitous presence of harmful chemicals (Arnold and Edwards, 2012; Dias et al., 2017) or pathogens (Rhoads et al., 2016; Salehi et al., 2020). It is quite worthy to find out what impact the tap water stagnation with such a long time and large scale has brought to tap water quality, especially to microbiological quality, which is essential for water safety.

A significant decline in the physicochemical and aesthetic quality of drinking water would occur if the tap water stagnates in the distribution pipelines (Caitlin et al., 2020; Salehi et al., 2020). In the absence of fresh tap water supplements, the chlorine residue in water decayed rapidly from 2.0 mg/L to 0.5 mg/L in 2 days, to 0 mg/L in 5 days (Ling et al., 2018). Moreover, the disinfectant residue was found to decay >140 times faster than in corresponding municipal water at highly stagnant taps (Rhoads et al., 2016). The dissolved oxygen (DO) in the water will also go down (Caitlin et al., 2020). Both of them cause water to shift from the oxidizing to reducing environment, which would lead to the breakage and detachment of pipe wall scaling and dissolving of heavy metals from the pipe materials as well as the long-term immersion works. Zhang et al. (2020) found that the turbidity of tap water could increase from 0.3 to 1.7 NTU in 48 h stagnation. The same stagnation duration could result in a Cu increase from below detected to 1370 and 1680 μg/L in kitchen and bathroom tap water, respectively (Zlatanovic et al., 2017). The increases of heavy metals including Fe, Mn, Cu, Ni, Cd were also observed in unlined cast pipe scales in a longer period of stagnation (132 h) in Zhengzhou, China (Li et al., 2020).

Microbial growth during water stagnation is well documented (Lautenschlager et al., 2010; Ling et al., 2018). For example, Chen et al. (2020) showed that the colony of total bacteria increased to >500 CFU/mL after short stagnation in water purifiers. Moreover, the stagnation was highly related to the occurrence of waterborne pathogenic microorganisms (Schwake et al., 2016; Kinsey et al., 2017). Several studies have identified the growth of *Legionella*, *Mycobacterium avium*, *Pseudomonas aeruginosa* during stagnation, although the curve may plateau (Bédard et al., 2016; Cooper et al., 2008; Haig et al., 2018). For example, in a hospital water pipe network with a retention time of 3-6 days, a high level of *Legionella* co-occurred with the high pipe scale debris detachment and low residual chlorine (Schwake et al., 2016), which matched well with the occurrence of Legionnaires disease.

However, all of the above-mentioned studies mainly focused on the analysis of water quality variation during short-term stagnation with a duration from several hours to several days (Zhang et al., 2020; Zlatanovic et al., 2017), which was not comparable with the stagnation during the current COVID-19 outbreak. Unfortunately, very limited information is available regarding this long-term stagnation on the tap water quality, especially on the microbiological parameters.

In this study, a field study was performed in University buildings located in a southeast city in China to elucidate the potential microbial risks and recovery periods of LTSW-induced contamination. Stagnation water samples were collected for three months (May to August 2020) from four types of college buildings, i.e., laboratory, canteen, teaching building, and dormitory buildings, after nearly four months of stagnation (middle January to May 2020). The samples were analyzed based on the physicochemical parameters, HPC, Taqman-based qPCR, flow cytometry (FCM), endotoxin analysis, and high-throughput sequencing. This study aims to address the knowledge gap of LTSW-induced microbial risks and provide useful advice on the safety control of drinking water.

2. Materials and methods

2.1. Sampling sites and water consumption

The studied facilities were three universities and one residential community in a city in Fujian province, Southeastern China. The campus water supply usages include everyday life (except cooking), cooking in the canteen, teaching, and lab work, etc. The detailed information of sampling site locations including frequency and water usage is shown in Table 1. All water samples were taken from the end of the water supply (tap water from the sink). University A was the main sampling site, of which the information is shown in Fig. 1. For university A, the winter vacation began in January 2020. Then, the sampling areas had seldom used tap water until graduate students returned to school in May because of the unexpected COVID-19 pandemic. In May 2020, the first month of sampling, the research and teaching activities were far from the normal levels since only a small part of students (about 1/12) returned to the campus (Fig. S1). The water consumption in the laboratory and teaching building of University A was almost zero. With the increased numbers of students returning to campus, the water supply was gradually restored to normal in August 2020. As additional sampling sites for both endotoxin and *Legionella pneumophila* indicators, the sinks of Universities B and C did not open during the sampling period. Their campus was closed for students and only opens for faculty and staff. By contrast, the residential community is located near University B, and the water supply in the community had not been interrupted and normal water consumption was maintained.

2.2. Water sampling

The study was mainly carried out in University A, and stagnation water sampling was performed over three months between May 2020 and August 2020. The water samples were taken in four types of public buildings in the university (Fig. 1). Besides, water samples were taken from University B, C and a residential community near University B, along with University A to determine endotoxin and *L. pneumophila* for the hazard assessment of long-term water retention. The residential community could be regarded as a negative control since its water supply was uninterrupted (Table 1). All stagnant water samples were collected from the tap at the sink. The water containers and tools were sterilized before sampling to eliminate the possible interference of bacterial contamination. Before sampling, the tap water will be flushed for 3 min. 10 L of tap water was collected at each sampling point, and the samples were transferred to the laboratory within 6 h (Guo et al., 2020).

2.3. Measurements of the physicochemical parameters of water samples

The chlorine residue, DO, oxidation-reduction potential (ORP), turbidity, temperature, and pH were determined in the field. The residual chlorine was measured using the N,N-diethyl-*p*-phenylenediamine (DPD) chlorine analyzer (HACH, USA). All other parameters were determined in situ by a multi-parameter water quality analyzer (HACH, USA).

The water samples were firstly filtered using a 0.45 μm polyethersulfone (PES) membrane (Millipore, USA) to remove the particles. Heavy metals were analyzed with inductively coupled plasma mass spectrometry (ICP-MS) (Agilent Technologies Inc. USA) after acidizing the water samples with nitric acid (5 mL/L) to pH < 2 (Sigma, USA). The total organic carbon (TOC) was detected by the TOC analyzer (Shimadzu, Japan). The nutrients, including the total dissolved nitrogen (TDN), total dissolved phosphorus (TDP), NO_2^-, NO_3^-, and NH_4^+, were determined by automatic nutrients analyzer AA3 (San++ analyzer, Germany). Specifically, TDN and TDP were oxidized by 4% alkaline potassium persulfate before analysis. The concentrations of the above parameters were calculated according to the pre-conFig.d standard curves.

Table 1
Description of sampling site locations including frequency and water usage. Water usage was based on system operator's observations and knowledge of building operations.

Sampling site	Description	Size	Sampling frequency	Usage
1	University A, laboratory, sink,	13,000 students 2600 faculty members	Two times per week then reduced to weekly	Low-frequency usage building
2	University A, canteen, sink, 2nd floor		Two times per week then reduced to weekly	Low-frequency usage building
3	University A, teaching building, washroom sink, 2nd floor		Two times per week then reduced to weekly	Low-frequency usage building
4	University A, dormitory, sink,		Two times per week then reduced to weekly	Low-frequency usage building
5	University A, dormitory, sink,		Once	Low-frequency usage building
6	Community near university B, sink	1027 families	Monthly, two times	Continuously water consumption building
7	University B, teaching building, washroom sink, 1nd floor	15,000 students 2590 faculty members	Monthly, two times	Long stagnation building
8	University B, dormitory, sink, 1nd floor		Monthly, two times	Long stagnation building
9	University C, laboratory, washroom sink, 1nd floor	20,000 students	Monthly, three times	Long stagnation building
10	University C, teaching building, washroom sink, 1nd floor	1100 faculty members	Monthly, three times	Long stagnation building
11	University C, dormitory, sink, 1nd floor		Monthly, three times	Long stagnation building

2.4. Microbial counting

1 mL of water samples was applied on nutrient agar (NA) (Hopebio, China) at 37 °C for 48 h to enumerate the total bacteria. The selective media for screening frequently-occurred pathogens was used to count *P. aeruginosa* (CN agar), *E. coli* (mTEC agar), *Enterococcus faecalis* (mEI agar), *Shigella* sp. (SS agar) (Hopebio, China), *Salmonella* sp. (BSA agar) (BD, USA) (USEPA1, 2002, 2006; Guo et al., 2020). In detail, the 100 mL water samples were concentrated by 0.45 μm PES membrane (Millipore, USA). Filtration membranes containing enriched bacteria were cultured on selected media. Two parallel groups were set for each sample. The specific preparation method and corresponding pathogen culture conditions are shown in Text S1.

Concerning endotoxin, its concentration was determined using an Endotoxin test Limulus Kit (Bioendo Technology Co., Ltd., China) following the manufacturer's instructions. The ratio of dead and alive bacterial cells was determined using FCM (Millipore Guava EasyCyte, USA) and LIVE/DEAD BacLight bacterial viability kit (Invitrogen, Inc. USA) according to Lin et al. (2017). SYTO 9 and propidium iodide (PI) were added into water samples with a final concentration of 2 μM and 20 μM, respectively. 5000 cells for each sample were counted after being stained for 20 min in the dark under 488 nm of light irradiation at room temperature.

2.5. Taqman probe-based qPCR

The water samples for qPCR and Illumina sequencing were concentrated by 0.22 μm PES membrane (Millipore, USA) and stored at -20 °C prior to DNA extraction. The genomic DNA was extracted using the FastDNA SPIN Kit (MP Biomedicals, USA) following the manufacturer's instructions. The Taqman-based probe was selected and designed (Table S1). Six representative waterborne pathogens (i.e., *E. faecalis*, *P. aeruginosa*, *E. coli*, *Salmonella* sp., *L. pneumophila*, *Shigella* sp.) were determined. The qPCR system with a final volume of 20 μL contained 10 μL of 2 × Taqman™ Gene Expression Master Mix (Thermo Fisher Scientific, USA), 0.05 μL of the probe (10 μM) (Sangon Biotech, China), 0.8 μL of

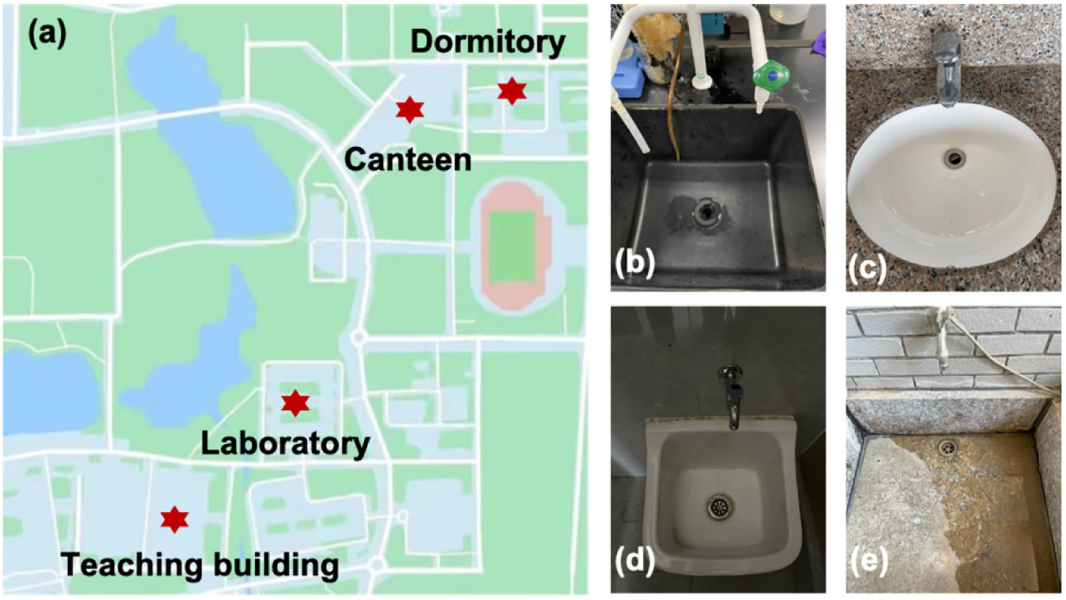

Fig. 1. Photographs of (a) sampling site information of the university A, (b) laboratory sampling site taps, (c) canteen sampling site taps, (d) teach building sampling site taps, and (e) dormitory sampling site taps.

each primer (10 μM), 2 μL of template DNA, and 6.35 μL of DNA-free water. The qPCR program consisted of a pro-denaturation step for 60 s at 95 °C, followed by 40 cycles of a denaturation step for 15 s at 95 °C, and an annealing step for 60 s at 60 °C using the ABI Q6 system (Life Technology, Singapore). Each target gene was run in triplicates. The standard curves were constructed from 10-fold serial dilutions of the plasmid standards that carry the target genes (Table S1). By comparison, the negative control used DNA-free water as the DNA template.

2.6. High-throughput sequencing (HTS)

The sequencing analysis of water samples was performed on the Illumina NovaSeq platform (Illumina, USA). Briefly, quality controlled genomic DNA (1 ng/μL) was amplified with the bacteria-specific primers (338 F/806 R) containing a barcode. The PCR products were detected by electrophoresis with 2% agarose gel and recovered using the gel recovery Kit (Qiagen, Germany). TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) was used to construct the library. After the library was qualified, NovaSeq6000 was used for sequencing (Novogene Science and Technology Co., Ltd., China).

2.7. Data analysis

The data were plotted in Prism 8.0 and processed with R studio, SPSS 16.0, and Prism 8.0. Uparse v7.0.1001 (http://www.drive5.com/uparse/) was adopted for OTU clustering. Mothur was used for species annotation according to the SSUrRNA database (http://www.arb-silva.de/), and BugBase was used for functional annotation. Continuous sampling was selected to characterize the repetition and stability of the data and reduce the experimental error.

3. Results and discussion

3.1. Increased Zn and Fe of stagnant water samples

In this study, eighteen physicochemical water quality parameters of the samples in University A were regularly measured, all of which could meet the tap water quality standard except the ones in Fig. 2. As seen, the long-time water retention resulted in the deterioration of two metals (Zn and Fe), residual chlorine, and turbidity. The other measured physicochemical parameters of stagnant water samples were summarized and are illustrated in Table S2. Long-term water retention did not have a significant impact on these indicators.

After long-time stagnation, the leaching of both zinc (Zn^{2+}) and iron (Fe^{2+}/Fe^{3+}) occurred in the plumbing system (Fig. 2). On the first day of the restarted water consumption, all of the samples suffered from the highest zinc and iron levels. The concentrations of zinc reached 7716.0, 6378.0, and 3082.0 μg/L in the samples from the laboratory, teaching building, and dormitory, respectively, which dramatically exceeded the Chinese national standard (i.e., 1000 μg/L). Similarly, the corresponding levels of iron were 1620.8, 700.4, and 716.9 μg/L, respectively, which were higher than the national standards of 300 μg/L. As the water usage got back to routine, the concentrations of zinc dropped rapidly and below the national standard within a week. Comparatively, the iron was restored even more quickly in three days. Compared with the other buildings, the concentrations of zinc and iron from canteen samples were lower than the national standard all the time, which may be attributed to the higher frequency and amount of water usage.

Cast iron and galvanized steel are widely used in the stem pipes in water distribution systems. It should be the main reason that the concentrations of zinc and iron in the stagnant water were elevated. Besides the debris detachment due to the long-term immersion, previous studies have confirmed that the metal ions could leak from the corrosion layer through the electrochemical reactions (Clark et al., 2015; Lasheen et al., 2008; Li et al., 2020). However, the iron and zinc concentrations in our study were much higher than those reported in Li et al. (2020) for metal release in 132 h of stagnation pipes (Fe: 190-260 μg/L, Zn: 1-10 μg/L), which might be attributed to that long retention time during the pandemic enhanced the corrosion and aquatic chemistry process. In addition, even the zinc and iron concentrations in the tap water could go down to the routine levels shortly in several days, special attention should be paid that the long water stagnation would probably have created corrosion "hot spots" in the pipe walls, which could pose long-term adverse impacts (Masters et al., 2015).

3.2. Decayed chlorine residue and increased turbidity of stagnant water samples

The qualified drinking water should contain residual chlorine to suppress bacterial regrowth. Because of the chlorine decay, the stagnant

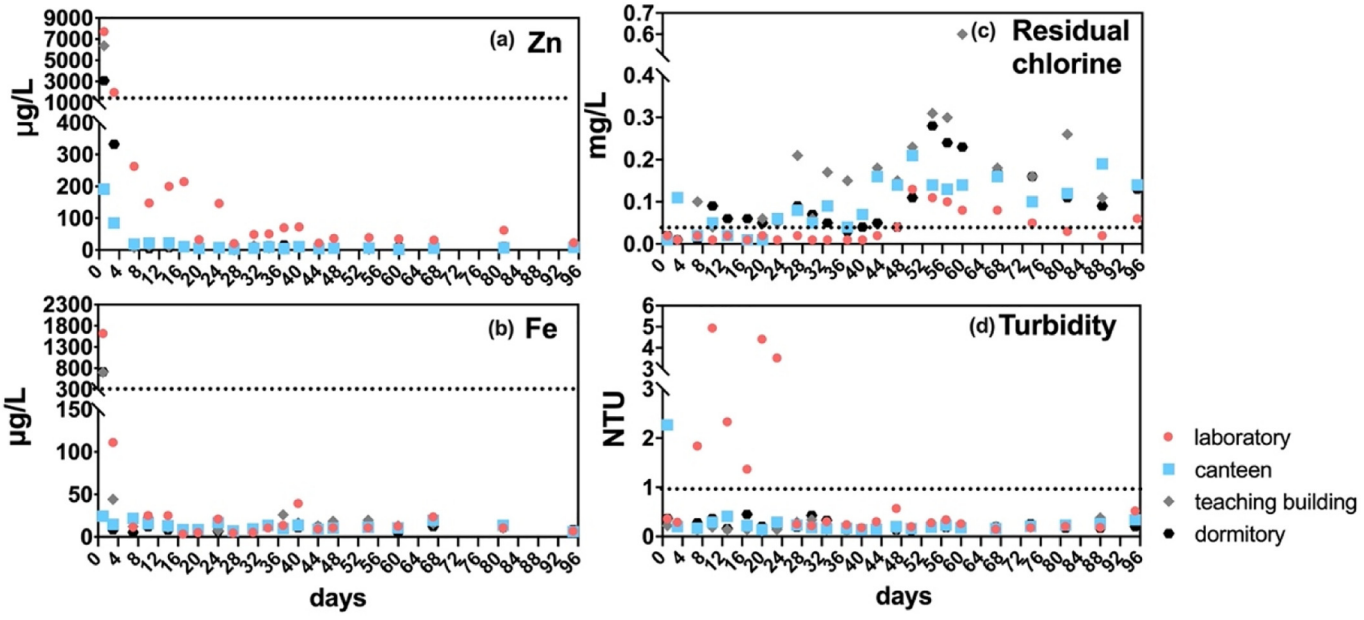

Fig. 2. The unqualified physicochemical parameters of different types of water samples collected during the stagnation. (a) Zinc, (b) iron, (c) residual chlorine, and (d) turbidity. Point "0" on the abscissa represents the starting point of the four-month water retention. The ordinate value of the dotted line is the national standard limit value.

water contained much lower chlorine levels than the fresh one. In the first week of the water re-consumption, the residual chlorine in all samples was below 0.05 mg/L, except for the canteen water samples (i.e., 0.05-0.11 mg/L) (Fig. 2(c)). While the retained water was subsequently consumed, more and more freshwater was supplemented into the water supply system. The residual chlorine concentrations gradually increased to the standard requirement (0.05 mg/L) in a period from 10 days to 48 days (the laboratory samples). According to the data issued by the local administration in December 2019 (before the COVID-19 pandemic) (Water Quality Bulletin, 2019), the level of residual chlorine in pipes of this area was 0.48 mg/L that was much higher. Residual chlorine has been well recognized as the most important factor for microbial inhibition (Caitlin et al., 2020; Zhang et al., 2020). The low level of residual chlorine in the pipe network is certainly a concerning safety hazard. In this study, the recovery duration of residual chlorine (especially laboratory water samples) was dramatically longer than the short period of water retention (Zhang et al., 2019, 2020), which suggested the occurrence of a much longer microbiological risk.

Except for the laboratory samples, the long-term water retention appears to exert much fewer effects on turbidity (Fig. 2(d)). The average turbidity of the dormitory, teaching building, and canteen was 0.30 NTU, 0.20 NTU, and 0.23 NTU, respectively, similar to those (<0.5 NTU) before the pandemic in this area. However, the maximum turbidity of the laboratory samples was 4.94 NTU, which exceeded the standard concentration (1 NTU) by nearly four times. It took 24 days to get back to the normal level. The water quality with high turbidity could probably provide suitable conditions for microbial attachment and biofilm formation (Schwake et al., 2016). In fact, this study also found that the high turbidity was accompanied by the detection of *L. pneumophilia*, which was discussed in detail in Section 3.5.

3.3. Significant increase in culturable bacteria

The influence of tap water supply recovery on the total culturable bacteria is reported in Fig. 3. It was observed that water retention would result in increased HPC values, which was consistent with the findings of previous studies (Pepper et al., 2004). However, compared with short-term stagnation (Chen et al., 2020), much more culturable bacteria were detected in this work due to the much longer retention. According to the issued data by the local administration, the HPCs were usually zero CFU/mL. But the measured bacteria in all four types of samples exceeded the Chinese national standard (100 CFU/mL). Because the scientific research activities were at a standstill during the epidemic, the laboratory samples had the most serious bacterial contamination with significantly higher HPC concentrations of up to 1.5×10^4 CFU/mL, two more magnitude orders than the standard. The recovery period for laboratory tap water was the longest. The HPC took about eight weeks to fall to the routine levels (no detection). Concerning three other sampling sites, the water usage was a little higher due to the daily life of the small number of persons on the campus. All of them fluctuated sharply during the first sampling month with several samples exceeding the standards, respectively. They merely differed from the laboratory samples in the much shorter recovery durations of 4-5 weeks.

Microbial growth depends on different environmental factors, such as temperature, disinfectant residue, nutrients, and pipe network material, etc. The correlations between the HPC concentration and residual chlorine was thus analyzed by using Spearman, in which a significant correlation ($P < 0.05$) was found between HPC and residual chlorine in the laboratory samples (Fig. 3). This phenomenon was also documented in a recent study on the HPC growth in the drinking water system (Lin et al., 2020). It was interesting that the HPC value always dropped to zero CFU/mL after one week when the residual chlorine

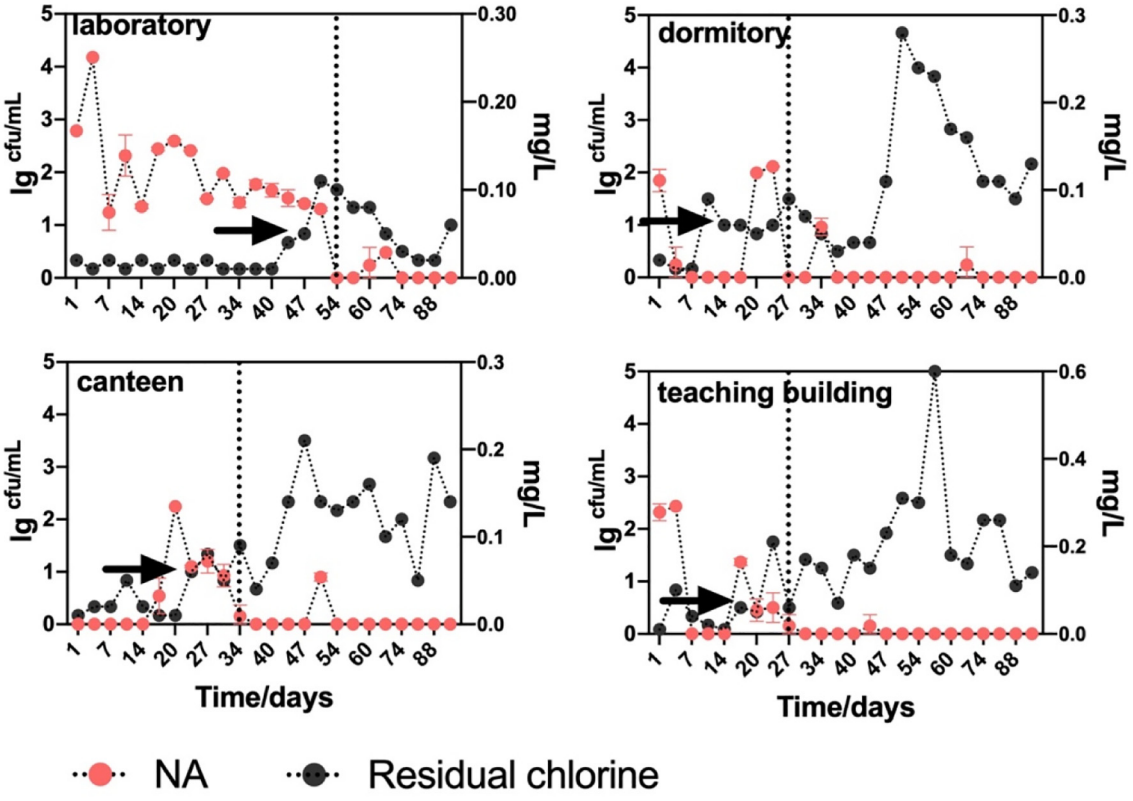

Fig. 3. HPC and residual chlorine levels during stagnation. The black arrow indicates that the number of culturable bacteria would not drop to 0 CFU/mL immediately after the increase of residual chlorine (>0.5 mg/L). The dotted line is the time point when the number of culturable bacteria drops to 0 CFU/mL. There was a lag relationship between culturable bacteria and residual chlorine.

level reached up to the national standard (0.05 mg/L) (see the black arrows in Fig. 3). Since the residue chlorine is easier and real-time to be detected, this time lag might be used as an indicator ahead of time for the microbiological safety of the tap water with a long stagnation.

3.4. Safety risks of pathogenic microorganisms

After long-time retention, the structures of the bacterial community in four different sampling sites were significantly different. The Principal Co-ordinates Analysis (PCoA) analysis showed that at the beginning of this study, four kinds of tap water samples were distributed in four different quadrants (Fig. S2), that is, their distribution was very dispersed. However, when the water supply resumed for about two months, microbial communities of the laboratory, canteen, and dormitory became uniformed. Among them, the community structure of canteen samples did not change significantly, which may be related to the constant tap water usage during the pandemic period. On the contrary, the teaching work always adopted the online mode, and only a small amount of flushing water was consumed, which might lead to the unstable results of microbial communities. This phenomenon suggested that the biological stability was gradually recovered in the tap water along with the water supply resumption.

Besides the total bacteria, the absolute abundance of six typical waterborne pathogens in the retained water samples was determined in this study since they were directly related to human health (Fig. 4). To obtain the occurrence of pathogenic bacteria more accurately, the Taqman-based qPCR method with higher sensitivity and specificity was adopted. Based on the standard curves (Table S1), the minimum detection limits (MDLs) of all pathogens were about 10 copies/mL, except for *Shigella* sp. and *L. pneumophila* whose MDLs were 10-100 copies/mL. As presented in Fig. 4, all pathogens were detected. The pathogen with the highest detection level (1.95×10^5 copies/100 mL) was from *L. pneumophila* in the laboratory samples. Comparatively, the highest levels for *Salmonella* spp., *Shigella* sp., *E. coli*, and *P. aeruginosa* were 1.70, 7.08, 7.24, 1.62×10^3 copies/100 mL, respectively. The recovery for these pathogenic microorganisms, i.e., below their MDL values, was about 2-5 weeks except for *L. pneumophila*. The absolute abundance of pathogens in different water samples was also higher when water quality parameters deteriorate. Guo et al. (2020) found that the levels of these pathogenic bacteria in the effluent of full-scale drinking water treatment plants (DWTP) remained at 0-10^2 copies/100 mL. The relatively high detection levels and long recovery period suggested that the health risks from the pathogenic bacteria in the retained water were much higher and should receive more attention.

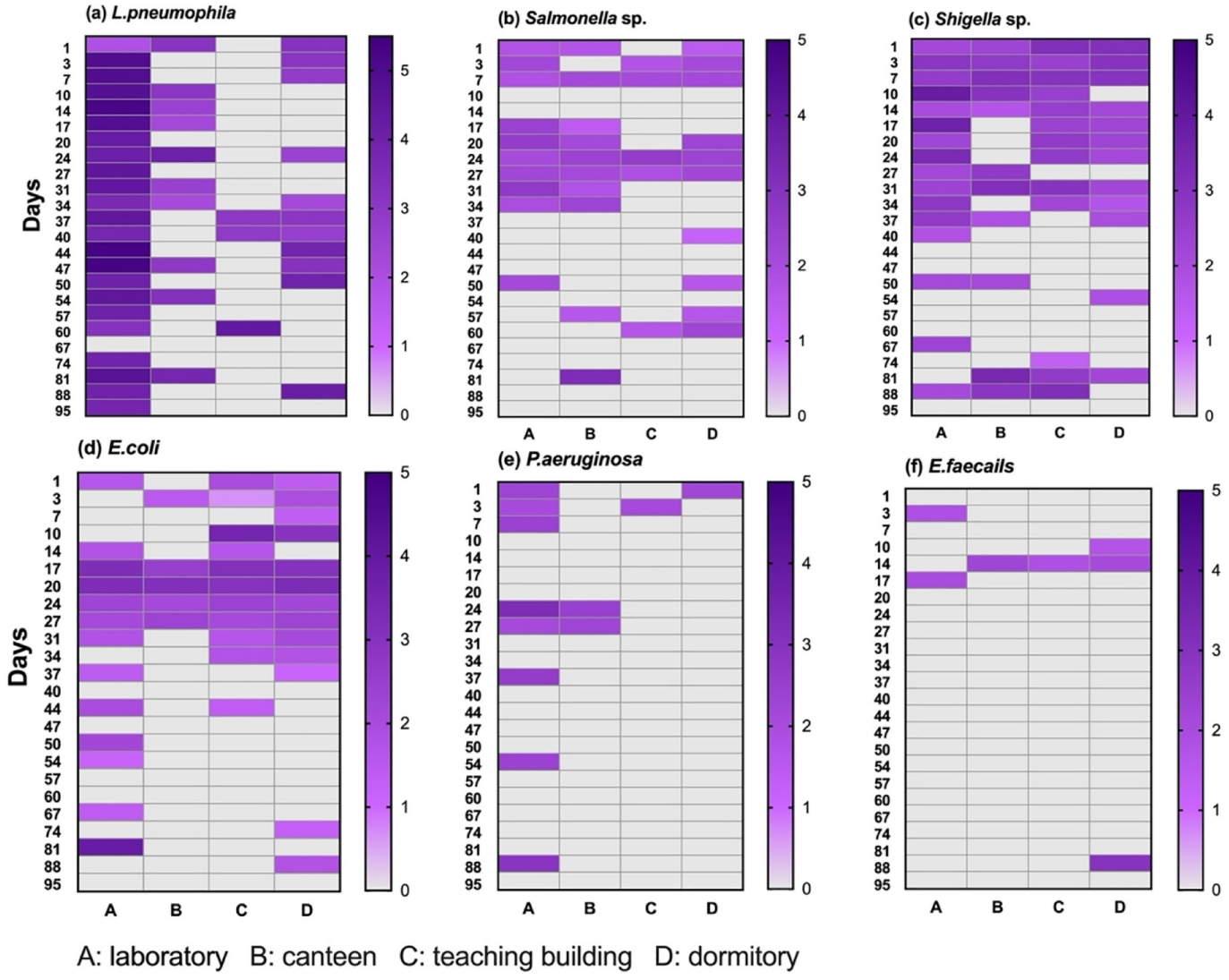

Fig. 4. The absolute abundance of typical pathogens by using Taqman-qPCR. (a) *L. pneumophilia*, (b) *Salmonella* spp., (c) *Shigella* sp., (d) *E. coli*, (e) *P. aeruginosa*, and (f) *E. faecalis*. Unit: $\log_{10}$ copies/100 mL.

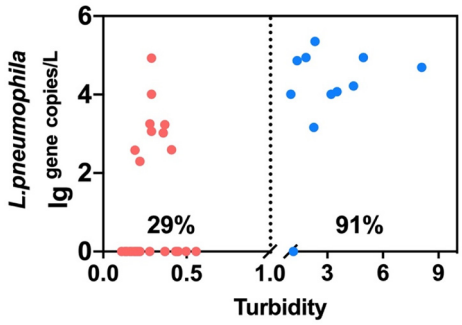

Fig. 5. The abundance of *L. pneumophila* at different levels of turbidity. Data were collected from the samples taken in the first month of University A, and samples from University B and C. Percentage indicated the detection rate of *L. pneumophila* under specific water quality conditions. For example, 91% means that under the condition of turbidity greater than 1 NTU, the detection rate of *L. pneumophila* is 91% in water samples.

3.5. Detection of L. pneumophila and its relationship with residual chlorine and turbidity

It is noteworthy to mention that *L. pneumophila* was continuously detected at high levels within three months during the recovery period of tap water supply in the laboratory samples (Fig. 4). Garrison et al. (2016) concluded that *L. pneumophila* was one of the most established causes of potable water-related disease outbreaks in the building plumbing systems. The outbreak of *L. pneumophila* was mainly connected with residual chlorine decay, iron release, and water stagnation. In fact, the decay of chlorine, which is the specific agent to inhibit microbial growth, was somewhat the result of the latter and could be accelerated by the latter (Lautenschlager et al., 2010; Ling et al., 2018). Likewise, Beer et al. (2015) and Shah et al. (2018) identified that depletion of residual disinfectant was the reason for Legionnaires disease outbreaks in public buildings. The above results suggested that chlorine maintenance should play a key role in the control of *L. pneumophila* in the stagnant water and its recovery. For example, shock disinfection within three weeks of planned occupancy was recommended for controlling remediation of *Legionella* colonization in the USA (ASHRAE Standards Committee, 2018).

In addition, it is of great significance to make early warning of *Legionella* in the LTSW environment. A co-occurring phenomenon was found for the detection of *L. pneumophila* and the initial turbidity of LTSW samples in this study. In particular, *L. pneumophila* was detected in 91% of the water samples with high turbidity (>1 NTU) (Fig. 5). *L. pneumophila* preferred to live in biofilm or other microbial aggregates in pipe walls or other media surfaces (Garrison et al., 2016; Proctor et al., 2018). In stagnant water, the mild hydraulic conditions and quick chlorine decay would accelerate biofilm formation. High turbidity implied higher numbers of particular matters such as the scaling debris, which was advantageous for biofilm formation. So the high turbidity might be used as an indicator of early warning of *L. pneumophila*.

3.6. VBNC bacteria in the stagnant water

In this study, the bacterial colonies with different morphology and colors were selected on the mediums, and a total of 86 strains were identified (Fig. 6(a)). It could be seen that *Sphingomona* was the dominant genera, accounting for 54.7% of all bacteria detected, followed by *Methylorubrum* at 13.3%. In addition, *Acinetobacter*, *Aeromonas*, and *Pseudomonas* were screened. *Sphingomona* is persistent and widely distributed in poor nutrition environments (e.g., mineral water or tap water) (Koskinen et al., 2000; Lee et al., 2001) and seldom present virulence or pathogenicity to human beings. However, a large amount of the bacterial cells might accumulate endotoxins when they were dead and decomposed, which would be discussed later. *Methylorubrum*, *Pseudomonas*, *Acinetobacter*, and *Aeromonas* were all reported as chlorine-resistant bacteria (Koskinen et al., 2000; Zhang et al., 2019; Zeng et al., 2020). Zhang et al. (2019) found that, with the increased secretion of extracellular polymers, *Methylorubrum* can form biofilms and thus resist the disinfectants. Overall, these pathogens were persistent in the LTSW environment, which require more attention. UV-based disinfection methods (e.g., UV/hydrogen peroxide and UV/peroxymonosulfate) were recommended for efficient inactivation of chlorine-resistant bacteria (CRB), therefore inhibiting the formation of biofilms (Zeng et al., 2020).

In this study, the top 35 genera in bacteria communities analyzed via HTS are listed in Fig. 6(c). *Phreatobacter* was the dominant genera, and its abundance was ranged from 17% to 81%, followed by *Sphingomona* with an abundance of 2%-17%. HTS results further confirmed that the proportion of pathogens in the total bacterial community was relatively low. The possible pathogens-HTS results were selected for the composition analysis of the pathogenic microorganisms (Fig. 6(b)). We found that results were much different from that culturable bacteria identification. In addition to the identified pathogenic bacteria in Fig. 6 (a), 16S rRNA gene fragments of the *E. coli*, *Helicobacter*, *Legionella*, *Mycobacterium*, *Staphylococcus*, *Streptomyces*, and other suspected pathogens were sequenced by the HTS analysis, while the culturable ones were not detected in the selective medium (Fig. S3).

Although the HTS results do not mean the existence of the active microbes, the risks of pathogenic bacteria should not be ignored, especially considering their entrance into the VBNC state. *Legionella* and *Mycobacterium* had the highest abundance, reaching 7.2×10^{-4} and 5.2×10^{-3}, respectively. These results were consistent with the Taqman-based qPCR method (Fig. 4). Based on the above analysis, VBNC pathogens occurred very likely in the LTSW environment and could evade the HPC detection standards. Similarly, Kinsey et al. (2017) reported that *P. aeruginosa* outbreak in a neonatal intensive care unit (ICU) was related to water retention in hospitals, which deserves more attention in terms of their potential health risks. Felföldi et al. (2010) observed a higher detection of positive samples for *Legionellae* using the qPCR technique compared to the cultivation method. Since the culturing methods as HPC are not applicable in detecting VBNC bacteria, the real infectious risks of the LTSW environment might be inaccurately estimated in many cases.

3.7. High-level endotoxin in LTSW

Endotoxin, composed of lipopolysaccharides, is a component of the cell wall of gram-negative (G^-) bacteria. It is also called “pyrogen”, which could cause fever, microcirculation disorder, endotoxin shock, and disseminated intravascular coagulation, etc. (Anderson et al., 2002; Liao et al., 2010). The endotoxin was mainly released by the G^- bacteria after death (Ren et al., 2019; Xue et al., 2019).

It could be concluded that the bacterial biomass kept relatively high levels in the stagnant tap water. During the beginning days of this study, the 16s rRNA genes were at log 7-9 copies/L, and most of the cultural bacteria were at log 1-4 CFU/mL. Since the stagnant period was over 4 months, which obviously exceeded the bacterial growth cycles in most natural and artificial circumstances, it could be inferred that the bacterial biomass would be in a pseudo-steady state (Chen et al., 2020), i.e., the dead bacterial cell numbers should be equivalent to the newly-divided cells, during most time of the stagnant. Therefore, another problem for LTSW was the accumulation of endotoxin produced by the in-situ lysis of the bacteria.

In this study, the levels of endotoxin in LTSW samples (the initial stage of water supply restoration) were analyzed (Fig. 7(a)). The results showed that the endotoxin levels were all increased in LTSW compared with the control group (i.e., tap water of always used). *t*-Test showed that the results were significantly different ($P < 0.05$), except for the D2 sample. FCM results showed that the proportion of dead bacteria in the LTSW (69.0%-96.7%) was significantly higher than that in the tap water always used (53.4%) (Fig. 7(b)). A greater proportion of

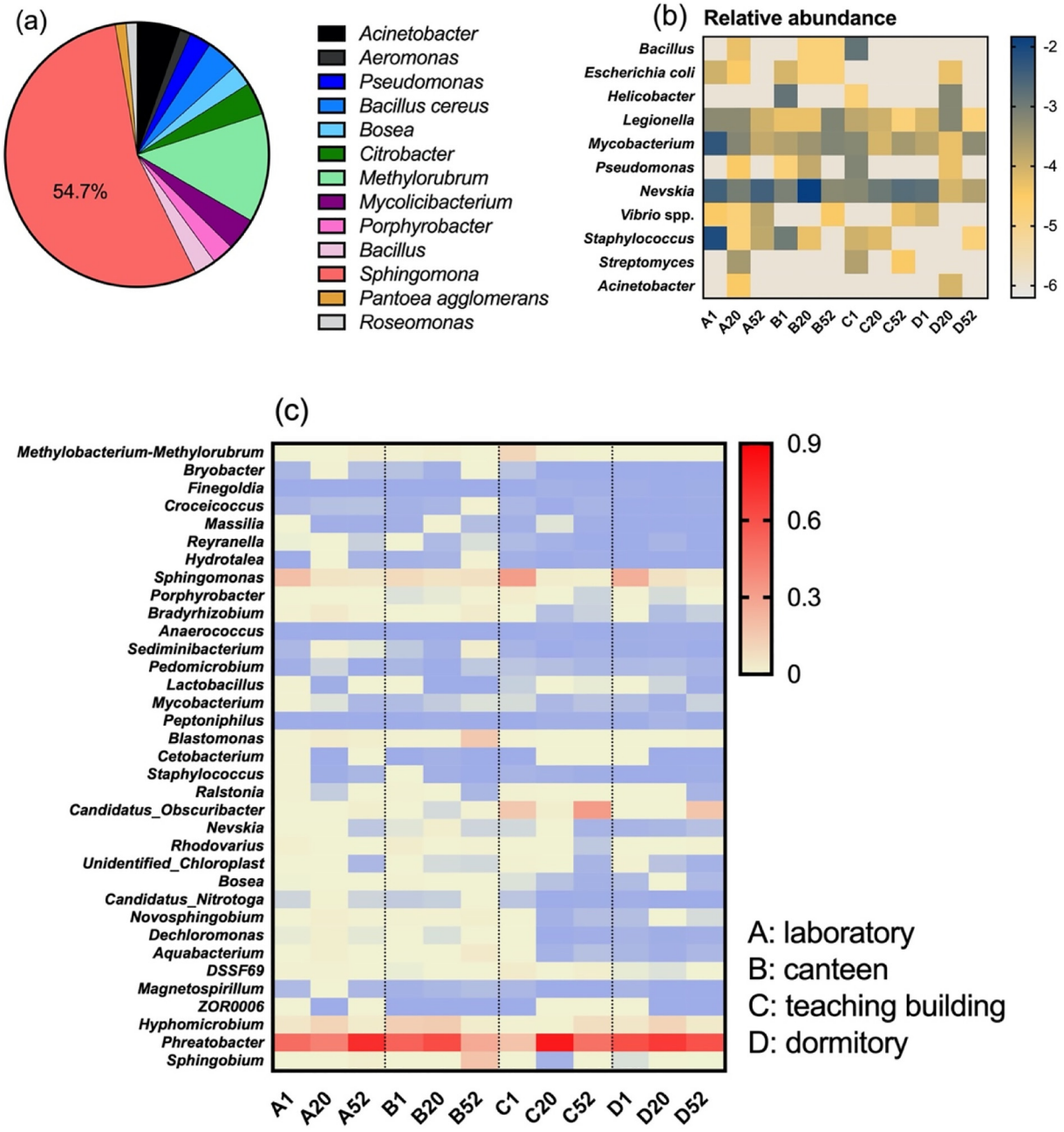

Fig. 6. Bacterial community composition of (a) culturable bacteria, (b) pathogens-HTS, and (c) top 35 genera-HTS of the samples from university A. Values indicate the $\log_{10}$-transformed relative abundance of bacteria in each genus.

bacteria in the retained water samples were in a state of membrane damage or even breakage. This further confirmed that high contents of endotoxin were related to the percentage of dead bacteria. Traditional biological indicators cannot reflect the contamination level of endotoxin. The presence of endotoxin in the LTSW is worthy of attention owing to its environmental persistence and pathogenicity.

3.8. Prevention of water quality issues during the COVID-19 pandemic

The University buildings impacted by COVID-19 had reduced or no water use for months. Our study confirmed that long-term water retention poses serious microbiological risks and thus prevention of water quality issues is essential. First of all, routine flushing is the most direct solution to pathogen control. Freshwater is regularly introduced to the pipeline network, and the stagnant environment cannot be formed, which helps prevent the problems. For the secondary water supply system of university buildings, attention should be paid especially to the cleaning of water tanks. It should be noted that recommissioning flushing could only reduce the levels of coliforms and heavy metals (Caitlin et al., 2020) but opportunistic pathogens can continue to grow (Hozalski et al., 2020), so it must be carried out with routine flushing during the COVID-19 pandemic. However, the frequency of routine flushing is difficult to determine. Factors such as plumbing design, the complexity of components, and the stored volume of water relative to water use need to be considered comprehensively. In the case of *Legionella*, Totaro et al. (2018) showed that effective control was achieved by maintaining a flushing frequency of 2 h. In addition, it is necessary to clean the water tank again for the university buildings with secondary water supply.

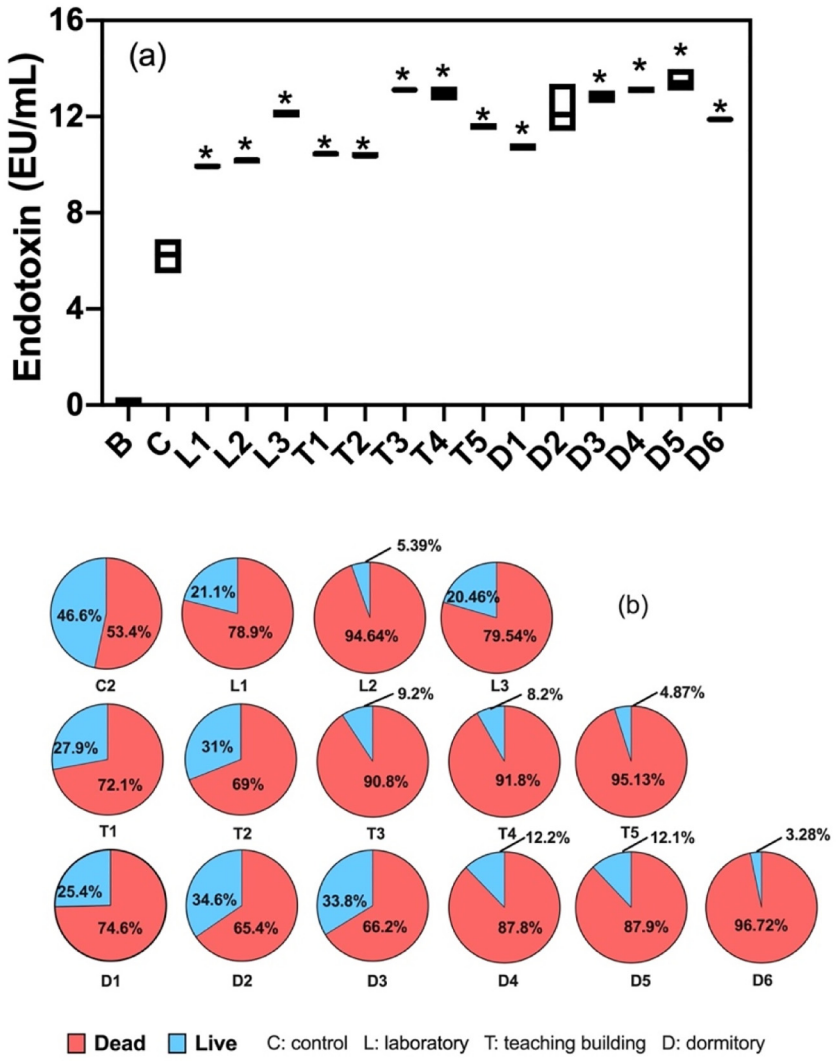

Fig. 7. (a) Content of endotoxin and (b) the proportion of dead and alive bacteria in different types of stagnant water. Data were collected from samples 6-11 in Table 1. B: blank control, water sample free of endotoxin; C: control (sample 6), the tap water came from the residential area nearly university; L: laboratory (sample 9); T: teaching building (sample 7, 10); D: dormitory (sample 5, 8, 9). "*" indicates a significant difference between the sample and the control group ($P < 0.05$).

It is important to maintain a disinfectant residual. By introducing a high level of disinfection for a short time, shock disinfection could effectively control pathogenic bacteria but must be weighed against the formation of disinfection by-products. Also, the water quality of University buildings should be monitored more frequently. Tap water with unqualified residual chlorine could be used for landscape irrigation, floor washing and other non-drinking purposes. If conditional, an automatic disinfectant device could be added to increase the delivery of disinfectant residual.

4. Conclusions

The global outbreak of the COVID-19 has led to the ultra long-term stagnation of tap water in public buildings such as those in university campuses all over the world. It was of common sense that stagnation would result in the overall deterioration of water quality including both the chemical and microbiological aspects. However, the impacts from such an ultra long-term stagnation, i.e. several months, were still unclear. Especially what microbiological risks the stagnation would bring was of great interest due to the increasing concerns from the public under the context of the epidemic. It was expected the conclusions below could answer at least part of the questions.

1) Long-term tap water retention resulted in the deterioration of water quality, while heavy metals (e.g., iron and zinc), turbidity, and chlorine were four key physicochemical parameters significantly exceeding the water quality guidelines, among which the latter two were closely connected to the microbial contamination.
2) Significant microbial growth occurred in the stagnant water, and the highest HPC of the samples reached two magnitude orders higher than the standards. It took 1-2 months to recover the bacterial levels to routine levels, which were much longer than the physicochemical parameters.
3) The microbiological risks in the LTSW were further confirmed by ubiquitous occurrence of six pathogenic species, among which *L. pneumophilia* had the highest detection frequency. However, these pathogens should probably be in the VBNC state due to the absence of the culturable ones. High turbidity (>1 NTU) might be an indicator for *L. pneumophilia*, suggested by their co-occurrence.
4) Endotoxin was a risk that has been overlooked in previous studies. A higher concentration of endotoxin (>10 EU/mL) in LTSW samples was detected, which resulted from the death of the high contents of the G^- bacteria.
5) Routine flushing and shock disinfection were recommended as the possible microbiological risks control methods during the COVID-19 pandemic.

CRediT authorship contribution statement

Chengsong Ye: Conceptualization, Methodology, Investigation, Software, Writing-original draft. **Xuanxuan Xian**: Sampling, Methodology, Visualization. **Ruihan Bao**: Sampling, Methodology. **Yiting Zhang**: Methodology, Visualization, Software. **Mingbao Feng**: Conceptualization, Writing-review & editing. **Wenfang Lin**: Writing-review & editing. **Xin Yu**: Conceptualization, Writing-review & editing, supervision.

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 data

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