In-situ features of LNA and HNA bacteria in branch ends of drinking water distribution systems

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ABSTRACT

The knowledge of bacteria with low or high nucleic acid (LNA or HNA, respectively) content was relatively limited in drinking water distribution systems (DWDSs). In the present study, LNA and HNA bacterial growth characteristics and effect of water physicochemical properties on these two subgroups were analyzed at the branch ends of a DWDS. The results showed that LNA bacteria were in the majority in tap water (67.2% ± 16.6%) and the specific growth rate (0.469 ± 0.022/d) was lower than that of HNA bacteria (1.116 ± 0.195/d). LNA and HNA bacteria showed different responses to water physicochemical properties. The LNA bacteria dominated the microbial community under relatively high conductivity conditions, and the HNA bacteria were predominant in high turbidity or alkalescence environments. The LNA bacteria growth rate was positively correlated to the assimilable organic carbon concentration. In addition, multivariate ordination illustrated that there were clear variances in water quality among outlets, which could result from different pipeline distances and hydraulic conditions.

Key words | assimilable organic carbon, bio-stabilization, drinking water distribution systems, flow cytometry, high nucleic acid content bacteria, low nucleic acid content bacteria

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INTRODUCTION

In China, it was reported that about 69% of drinking water hygienic accidents (e.g. abdominal pain, diarrhea and enteritis) were attributed to biological pollution (Li *et al.* 2007). Current standard methods of microbial examination of drinking water are based on cultivation, e.g. heterotrophic plate counts (HPCs) and multiple tube-fermentation. However, most of microorganisms in natural environments are viable but non-culturable, and only less than 1% of the microorganisms can be cultivated by HPCs (Wang *et al.* 2010a). Meanwhile, conventional methods are not suitable for prompt monitoring of microbial changes in drinking water due to their long examination duration, and may lead to delays in safety management (Besmer *et al.* 2014). In addition, water quality of end consumers would change during the transport process in drinking water distribution systems (DWDSs) due to the difference of hydraulic retention time and pipeline materials (El-Chakhtoura *et al.* 2015; Roeselers *et al.* 2015; Abokifa *et al.* 2016; Liu *et al.* 2016a). Microorganisms would be massively propagated if the concentration of assimilable organic carbon (AOC) was too high in drinking water, where opportunistic pathogens would cause hygienic problems (Prest *et al.* 2016). Excessive growth of bacteria would also result in biocorrosion of water supply pipelines and further lead to secondary pollution of drinking water (Holinger *et al.* 2014; Van Nevel *et al.* 2016). Therefore, it is essential to understand and control the microbial growth at the branch end of DWDSs in drinking water quality management (El-Chakhtoura *et al.* 2015).

Flow cytometry (FCM) has been applied recently to analyze microbial dynamics in drinking water, as a new monitoring method (Wang *et al.* 2010a; Besmer *et al.*

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2014). Bacteria would cluster into two distinct subgroups, low nucleic acid content (LNA) bacteria and high nucleic acid content (HNA) bacteria, when analyzed by FCM in combination with fluorescent staining (Wang *et al.* 2010a). LNA bacteria, playing a critical role in aquatic ecosystems, are considered to be oligotrophic and dominant in low nutrient environments (Solic *et al.* 2009). Drinking water is a typical oligotrophic environment where the amount of total organic carbon is usually less than 1 mg/L (Lautenschlager *et al.* 2013; Kelly *et al.* 2014). It was proposed that there may be microenvironments where LNA and HNA bacteria could co-grow in drinking water (Prest *et al.* 2013). Particularly, oligotrophic bacteria may maintain high abundance in drinking water, which would impose a potential threat to human health and safety (Liu *et al.* 2013a). Hence, it is necessary to investigate the bacteriology on LNA and HNA features and their difference in DWDSs networks.

Here the LNA and HNA bacteria were analyzed at different outlets from a DWDS where the residual chlorine is no less than 1.4 mg/L in the effluent of drinking water treatment processes. The aims of the present study were: (1) to analyze the LNA and HNA bacteria content and compare their in-situ co-growth characteristics in branch ends of DWDS; (2) to illustrate the effect of water physicochemical properties on LNA and HNA bacteria.

MATERIALS AND METHODS

Microorganism detection

Drinking water samples were taken from four main outlets of the DWDS in TEDA College (Tianjin, China). It covers a field of 70,000 m^2 and comprises teaching and living districts. Chlorination was used as one of the main disinfection methods in the drinking water process. The residual chlorine is no less than 1.4 mg/L at the beginning of the DWDS. All outlets were supplied from the same water treatment plant and three replicates of 2 L water samples were collected in each outlet at room temperature and the sampling water was analyzed immediately according to the following methods.

Heterotrophic plate counts

The cultivation medium was composed of peptone (10 g/L), beef extract (3 g/L), NaCl (5 g/L) and agar (20 g/L) with pH 7.4 to 7.6. The medium was sterilized at 121°C for 20 min. 100 μL of water sample was transferred to a petri dish containing 15 mL sterilized medium. The plates were incubated at 37°C for 48 hour so the colonies can be counted. All measurements were done in triplicate, and three blanks were set as the control.

Multiple tube-fermentation-most probable number measurements

The cultivation medium was composed of peptone (20 g/L), beef extract (6 g/L), lactose (10 g/L), NaCl (10 g/L), bromocresol purple (16 g/L ethanol-soluble, 2 mL), pH 7.2–7.4. The medium was sterilized at 121°C for 20 min. 10 mL of water sample was transferred to a tube containing 10 mL sterilized medium. The samples were incubated at 37°C for 24 h. All measurements were done with five replicates.

FCM analysis

Staining and flow cytometric measurements were performed following the procedures as described previously (Lautenschlager *et al.* 2013). Each sample water (1 mL) was stained with 10 μL/mL staining solution, a mixture of 100 times diluted SYBR Green I in dimethyl sulfoxide and 20 mmol/L propidium iodide (Invitrogen, USA) at a volume ratio of 50:1. The samples were then incubated in the dark for 25 min before measurement. FCM measurements were performed on a CyFlow Space (Partec, Germany). Green and red fluorescences were collected at 520 nm and 615 nm, respectively. The specific instrumental gain settings were as follows: side scatter (SSC) = 280, FL1 = 410, FL3 = 700, and collected as logarithmic (4 decades) signals. LNA and HNA bacterial concentrations were counted separately and the respective geometrical means of green fluorescence (FL1) and SSC were calculated. All samples were measured in triplicates. In order to be measured by FCM, water samples were diluted in cell-free Milli-Q water and the concentration was always lower than 2×10^5 cells/mL. The detection limit was

under 500 cells/s with an average standard deviation of 5% (Liu *et al.* 2016b).

Growth characteristics assay

Carbon-free vials were prepared as described previously (Vital *et al.* 2012). Triplicate water samples were incubated at 30°C for 3 days. LNA and HNA bacterial concentration of the native microbial community were determined before and after incubation by FCM as described above. The specific growth rate (μ) was calculated using the following equation:

$$\mu = \frac{\mathrm{Ln}(C_t) - \mathrm{Ln}(C_0)}{\Delta t}$$

where C_0, C_t are the bacterial concentration (cells/mL) measured before and after incubation, and Δt is the time interval (3 d, in the present study).

A simplified version of the AOC assay was adopted to assess microbial regrowth potential (Vital *et al.* 2007). The net growth cell was obtained by converting the AOC concentration using the following equation:

$$\mathrm{AOC}[(\mu gC)/L] = \frac{(C_t - C_0)\mathrm{cells/L}}{1 \times 10^7 \mathrm{cells}/(\mu gC)}$$

where C_0, C_t are the bacterial concentration (cells/mL) measured before and after incubation.

Physicochemical measurements

Turbidity, chrominance, pH and conductivity were measured using an YSI EC300 Water Quality Sonde, and ammonia nitrogen (NH_3), nitrite (NO_2), phosphate (PO_4) and residual chlorine (RCl) were assayed as described in our previous studies (Ma *et al.* 2016). Heavy metals (Cd, Pb, As and Cr) were measured using inductively coupled plasma mass spectrometry. Standard samples of heavy metal with different concentration gradients were prepared and Milli-Q water was used as the control sample to generate standard curves. Water samples were filtered through a 0.45 μm pore size filter, and directly used to measure the heavy metal concentration. All measurements were done in triplicate.

Data analysis

To illustrate the effect of water physicochemical properties on LNA and HNA bacterial characteristics (including the specific growth rate and proportion of LNA and HNA) and variance of water quality, a multivariate constrained ordination technique (redundancy analysis, RDA) was performed by R statistical software using the package 'Vegan' (http://www.r-project.org/) after data centralization and standardization, and the Monte Carlo test was used to examine the significance of correlations between LNA and HNA bacterial characteristics and water physicochemical properties in RDA. One-way analysis of variance was conducted to compare data variation.

RESULTS AND DISCUSSION

LNA and HNA bacterial growth characteristics

FCM analysis revealed that there were LNA and HNA bacteria groups in drinking water (Figure 1(a)), which is consistent with previous reports (Prest *et al.* 2013; Besmer *et al.* 2014). LNA bacteria were the predominant community (67.2% ± 16.6%), which was significant higher than that of HNA bacteria ($P < 0.01$) (Figure 2(a) and 2(b)). It could be attributed to LNA bacterial characteristics (i.e. small cell sized and oligotrophic). Most of the source water of DWDSs came from lake and river, where LNA bacteria were widely distributed (Besmer *et al.* 2014). The small cell

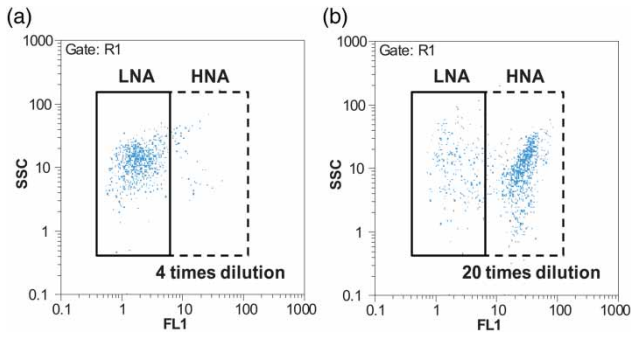

Figure 1 | Flow cytogram of LNA and HNA bacterial community (a) before and (b) after 3 days' incubation. FL1 and SSC stand for forward scatter and side scatter signal, respectively.

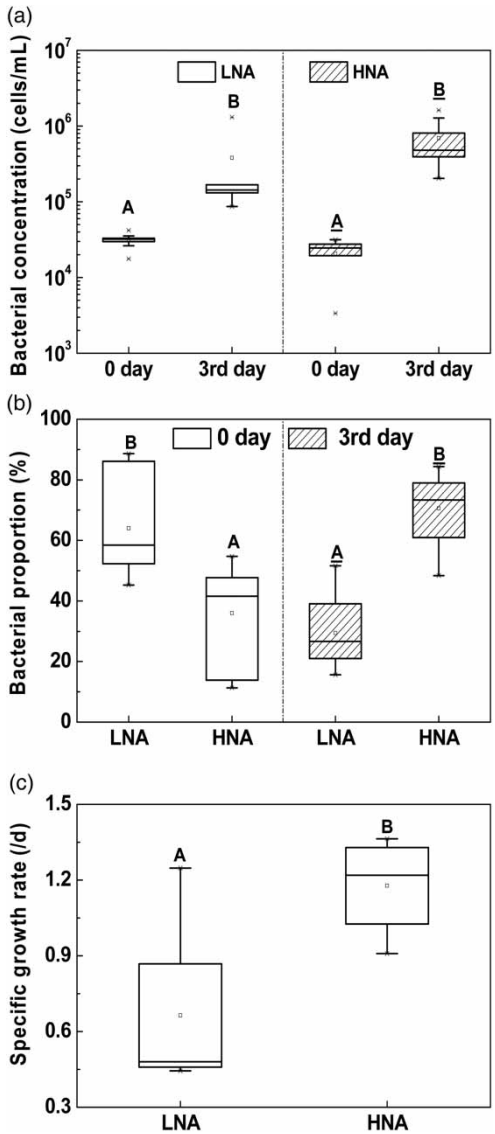

Figure 2 | The (a) concentration and (b) percentage of LNA and HNA bacteria before and after 3 days' incubation, and (c) their specific growth rate. Different capital letters indicate significant differences at the 0.01 level.

size of LNA bacteria could enable them escaping from 0.22 μm pore-size-microfiltration based sterilization procedure (Wang *et al.* 2008). Moreover, it was reported that HNA bacteria were more susceptible to chlorine dioxide and permanganate than LNA bacteria (Ramseier *et al.* 2011). It was suggested that LNA and HNA cell membranes have different chemical functional groups, which could make the LNA bacteria resistant to oxidant damage (Lee & von Gunten 2010) and survive in the sterilization process and outnumbered the HNA.

After incubation for 3 days at 30°C, the bacterial community changed dramatically and the HNA bacteria became predominant in the community (69.3% ± 11.1%) (Figure 1(b), Figure 2(a) and 2(b)). HNA bacterial concentration was higher than that of LNA bacteria after the incubation ($P < 0.01$), which was different to the bacterial growth situation in the pipe of DWDSs, i.e., initial incubation stage. The reason may be that accumulations of loose deposits create nutrient-rich microenvironments (Liu *et al.* 2014). As LNA bacteria are reported to be oligotrophic and cannot grow well in high nutrient condition, eutrophic HNA bacteria would become predominant (Vital *et al.* 2012). Therefore, although both LNA and HNA bacterial concentrations significantly increased after incubation ($P < 0.01$) (Figure 2(a) and 2(b)), the specific growth rate of LNA bacteria (0.469 ± 0.022/d) was lower than that of HNA (1.116 ± 0.195/d) ($P < 0.01$) (Figure 2(c)). The results were consistent with a previous investigation carried out in the natural aquatic ecosystems (e.g. lake) (Nishimura *et al.* 2005). Meanwhile, the results showed that not only bacterial concentration changed, but also bacterial community subgroups composition varied. The flow cytometric parameters of the total bacterial community shifted along with changes of the percentage of LNA and HNA. Green fluorescence (FL1), which reflects the cell DNA content, had a significant increase ($P < 0.01$) after 3 days' incubation. In contrast, SSC, which reflects cell size, exhibited no obvious changes ($P > 0.1$) (Figure 3). These findings suggested that microbial community in drinking water could change after long retention time.

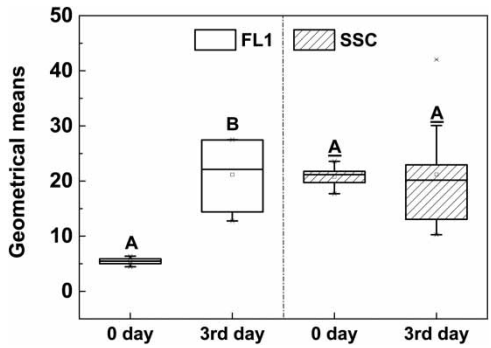

Figure 3 | The changes of green fluorescence (FL1) and side scatter (SSC) in the total bacterial community before and after 3 days' incubation. Different capital letters indicate significant difference at the 0.01 level, and letters without and with underline represent differences before and after the 3 days' incubation, respectively.

Effect of water physicochemical properties on bacterial community

The physicochemical properties of water samples from different outlets is shown in Table 1. The Monte Carlo test showed that RDA axes 1 and 2 were significant in elucidation of the correlations between bacterial characteristics and water physicochemical properties ($F = 24.7$, $P = 0.008$). The results revealed that water physicochemical properties had different effects on the LNA and HNA bacteria in tap water (Figure 4). It showed that the HNA bacteria (HNA%) were predominant in relative high turbidity or chrominance tap water. The reason could be that the formation of eutrophic microcosm around particulate matter would promote growth of HNA bacteria in tap water with high turbidity or chrominance (Vital *et al.* 2012; Liu *et al.* 2014). In the present study, FCM-live (LIVE) bacteria concentration showed a strongly negative correlation to cadmium (Cd) (Pearson's $R = -0.964$, $P < 0.01$), arsenic (As) ($R = -0.842$, $P < 0.01$) and conductivity ($R = -0.980$, $P < 0.01$). A negative correlation was also observed between these parameters and HNA% (Figure 4). It indicated that those heavy metals and conductivity would restrict the microbial activity and growth. In contrast, the LNA bacteria (LNA%) were positively correlated to the metal concentrations (i.e. arsenic ($R = 0.889$, $P < 0.01$) and cadmium ($R = 0.977$, $P < 0.01$)) and conductivity ($R = 0.983$, $P < 0.01$) (Figure 4). It suggested that the survival rate of LNA was higher than HNA and LNA dominated in the relative high heavy metal content or conductivity tap water. The reason could be that bacteria exhibited different susceptibility to metals (Tada *et al.* 2001).

Furthermore, the results showed that LNA bacterial specific growth rate was positively correlated to the AOC concentration (Pearson's $R = 0.987$, $P < 0.01$) (Figure 4). In contrast, no significant correlation between HNA growth rate and AOC was observed (Figure 4). For instance, the AOC level was similar between Outlet 1 and 4 (22.5 vs. 30.6 μg C/L), but HNA specific growth rate in Outlet 1 (1.279 ± 0.069/d) was significantly higher than that of Outlet 4 (0.896 ± 0.033/d) ($P < 0.01$). This may be due to the variation in carbon utilization ability among HNA bacterial community. Furthermore, factors including disinfection concentration, pH, temperature, etc., could also affect the correlation between bacterial growth rate and AOC concentration in DWDSs (Vital *et al.* 2007; Liu *et al.* 2015). Hence, more water samples from different outlets and in different DWDSs should be tested in order to have a better understanding of bacterial growth characteristics in drinking water.

Table 1 | Physicochemical properties of water samples from different outlets

	Water samples			
Physicochemical properties	**Outlet 1**	**Outlet 2**	**Outlet 3**	**Outlet 4**
pH	7.62 ± 0.10	8.31 ± 0.01	8.29 ± 0.04	8.23 ± 0.05
Turbidity (NTU)	0.195 ± 0.072	2.420 ± 0.207	2.206 ± 0.117	2.491 ± 0.097
Water chroma (PCU)	1.585 ± 0.934	28.862 ± 0.319	21.165 ± 1.207	19.878 ± 0.051
Nitrite (mg/L)	UD	0.032 ± 0.001	0.008 ± 0.000	0.010 ± 0.001
Phosphate (mg/L)	0.008 ± 0.001	0.169 ± 0.002	0.018 ± 0.002	0.032 ± 0.006
Residual chlorine (mg/L)	0.055 ± 0.002	0.062 ± 0.002	0.055 ± 0.003	0.083 ± 0.001
Ammonia nitrogen (mg/L)	1.243 ± 0.022	1.082 ± 0.022	2.346 ± 0.015	0.799 ± 0.005
Conductivity (μS/cm^2)	394.0 ± 1.0	365.0 ± 2.8	364.5 ± 0.7	361.0 ± 1.4
Cd (μg/L)	0.081 ± 0.007	0.018 ± 0.005	0.023 ± 0.005	0.017 ± 0.007
Pb (μg/L)	0.234 ± 0.011	UD	UD	UD
As (μg/L)	0.492 ± 0.056	0.373 ± 0.024	0.393 ± 0.020	0.372 ± 0.021
Cr (μg/L)	4.829 ± 0.053	5.981 ± 0.060	6.795 ± 0.102	5.418 ± 0.092

UD = undetectable.

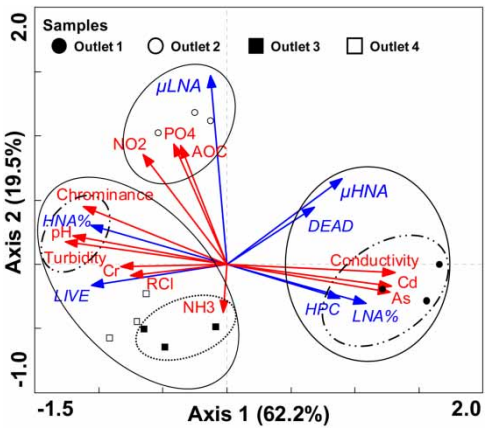

Figure 4 | Redundancy analysis plot of LNA and HNA bacterial characteristics with water physicochemical properties. Red and blue arrows represent variables of water physicochemical properties and bacterial characteristics, respectively (NH_3, ammonia nitrogen; NO_2, nitrite; PO_4, phosphate; R_{Cl}, residual chlorine; μLNA and μHNA, the specific growth rate of LNA and HNA, respectively; LIVE and DEAD, live and dead bacterial concentration; HPC, heterotrophic plate counts; LNA% and HNA%, the contribution of LNA and HNA in total FCM-bacteria, respectively). Please refer to the online version of this paper to see this figure in colour: http://dx.doi:10.2166/aqua.2017.108.

The results showed that the water quality had great variance among different outlets where outlets was clustered together based on their location and linked to different variables in Figure 4 (indicated by corresponding individual cycles, Pb concentration was not shown as it was below the detection limit except in Outlet 1), it was reflected that each outlet corresponded to different individual physicochemical environments. The results indicated that the water had experienced complex changes during the transport process in the water supply network after leaving the treatment plant. The variations could result from variations in hydraulic conditions (e.g. flow velocity and residence time) (Abokifa *et al.* 2016). When flow rate and pressure change, biofilm detachment and loose deposits may occur in DWDSs (Liu *et al.* 2013b), leading to deterioration of water quality during distribution (Nguyen *et al.* 2012; Douterelo *et al.* 2016). It was reported that bacteria associated with pipe wall biofilm and loose deposits contribute to over 98% of the total bacteria in DWDSs (Liu *et al.* 2014).

AOC and residual chlorine were considered as the main indicators for microbial regrowth in DWDSs (Srinivasan & Harrington 2007). AOC concentration showed significant variation ($P < 0.001$) among different outlets, which may be a result of different transportation distances. It was suggested that the acceptable AOC concentration for biological stability was 10.9 μg C/L under a minimal chlorine residual condition (0.05 mg/L) (Ohkouchi *et al.* 2013). When AOC was higher than 10.9 μg C/L but lower than 100 mg C/L, it would require maintaining residual chlorine (higher than 0.5 mg/L of free chlorine or 1.0 mg/L of chloramines) to restrain coliform regrowth in drinking water (Hammes *et al.* 2010). In the present study, AOC concentration ranged from 22.5 to 258.6 μg C/L and the level of residual chlorine concentration was about 0.06 mg/L in the outlets. Hence, there is a potential risk of bacterial overgrowth in the tested outlets due to their low residual chlorine concentration and high AOC concentration.

Comparison of microbial detection methods

Rapid and accurate detection of microorganisms is critical to ensure the safety and quality of drinking water (Besmer *et al.* 2014; Van Nevel *et al.* 2016). In the present study, the microbial concentration was measured by HPC, multiple tube-fermentation and FCM separately. Figure 5 shows that the standard plate-count bacteria (HPCs) was less than 100 CFU/mL (blue line) in all outlets, and total coliforms were not detectable based on the most probable number of multiple tube-fermentations (data not shown). The index of plate-count bacteria and total coliforms met the national standard (the microbial colonies were less than 100 CFU/mL and no coliforms were detected) of drinking water quality (Ministry of Health of the People's Republic China & SAC China 2006). However, FCM results showed that live bacterial concentration ranged from 0.36 ×

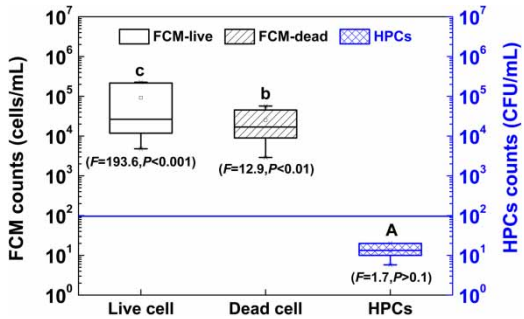

Figure 5 | Comparison of microbial biomass index between FCM and HPCs. Blue line represents the Chinese drinking water standard of total bacterial colonies. Different capital and lowercase letters indicate significant differences at the 0.01 and 0.05 levels, respectively. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/aqua.2017.108.

10^5 to 2.26×10^5 cells/mL accounting for 67.4%–83.91% of total cells, which was significantly higher than the concentration of dead bacteria (1.73×10^4–5.68×10^4 cells/mL) ($P < 0.05$), and HPC-bacteria (cultivable bacteria) concentration was less than 0.1% of FCM-live bacteria concentration ($P < 0.01$). In addition, HPC concentrations had no significant variations ($F = 1.7$, $P > 0.1$) while significant difference was observed in FCM-live ($F = 193.6$, $P < 0.001$) and FCM-dead ($F = 12.9$, $P < 0.01$) bacteria among the tested outlets. It demonstrated that the conventional HPC method significantly underestimated the abundance of microorganisms, which may impose risk in the safety assessment of drinking water. Hence, it is necessary to modify and improve the present standard microbial examination methods and quantity standards for drinking water.

Compared with the HPC method, FCM is more efficient and irrespective of culturability. Hence, it can be applied to real time microorganism monitoring in drinking water treatment and supply systems to reduce the risk of water quality in DWDSs (Lautenschlager *et al.* 2013; Van Nevel *et al.* 2016). Furthermore, it was reported that some readily culturable bacteria may enter a temporarily non-culturable state in response to certain stimuli. FCM has overcome one major obstacle on cultivability in microbiology studies, which has made the observation of unculturable bacteria feasible (Wang *et al.* 2010a). Meanwhile, FCM can be used to evaluate the effect of disinfection processes by analyzing the microbial distribution (live and dead bacteria) in drinking water treatment plants (Wang *et al.* 2010b).

CONCLUSIONS

In this study, it was found that typical clusters of LNA and HNA bacteria were observed in the branch ends of DWDSs, an LNA bacteria was the predominant community.

LNA and HNA bacteria showed different responses to water physicochemical properties. The LNA bacteria dominated in relatively high heavy metal (Cd, As) or conductivity conditions, and the HNA bacteria were the predominant in high turbidity or alkalescence environments.

The specific growth rate of LNA bacteria was lower than that of HNA in drinking water, and was positively correlated to AOC concentration.

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