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Optimization of ultrasonic parameters for effective detachment of biofilm cells in an actual drinking water distribution system*#

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Abstract: It is important to obtain a considerable quantity of DNA from oligotrophic environments such as a drinking water distribution system (DWDS) to study microbial communities by molecular biotechnology, and DNA yield is always one of the biggest problems when performing metagenomic sequencing on drinking water samples. To obtain as many microbes as possible, ultrasound has been widely used in cell detachment, but studies on the optimal ultrasonic parameters for biofilm in DWDS have rarely been seen. The effects of three ultrasonic parameters, including power, duration, and the number of ultrasound treatments (USTs) on the selected monoculture bacteria (*Pelomonas sp.*) biofilm were studied first. Then the optimal values of each ultrasonic parameter were initially determined. Based on these values, three levels of each ultrasonic parameter were selected, and then an orthogonal experiment was conducted to further study drinking water biofilm, and finally the optimal ultrasonic parameters for the effective separation of biofilm cells in DWDS were determined. The results showed that the optimal ultrasonic power, duration, and the number of USTs are 13 W, 1 min, and 15, respectively. A 20-min interval is needed between two USTs. The present optimal UST, which does not lose DNA quality, can increase the amount of extractable DNA by at least 4.78 times compared to samples without UST. This study provides a pretreatment methodology for extracting more and reliable DNA from biofilm in DWDS, and can better solve the problem of DNA collection in oligotrophic environments.

Key words: Biofilm; Drinking water distribution system (DWDS); DNA yield; DNA quality; Ultrasound treatment (UST)
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1 Introduction

Biofilm in drinking water distribution system (DWDS) is the key to understanding and controlling the microbiological risk of drinking water (Zhou et al., 2009; Liu et al., 2017). Many laboratorial reactors and scaled DWDS were popular in previous studies on DWDS biofilm (Lehtola et al., 2006; Douterelo et al., 2013; Gomes et al., 2014; Zhu et al., 2014; Ji et al., 2017), but the process of model simplification would lead to distortions of test results, so that a representative biofilm sample should be directly obtained from the actual DWDS. Given that the majority of microbes existing in the environment are not culturable, further studies of microbial communities are mostly based on environmental DNA by

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cultural-independent methods. It is essential to obtain as many cells as possible for analysis, especially from oligotrophic environments such as DWDS. Using the instructions of TruSeq DNA Exome Library Prep Kit (Illumina, USA), a DNA library for metagenomic sequencing can be prepared using as little as 0.1 μg of DNA. However, to ensure accuracy of the data, general sequencing companies require the DNA amount to be more than 2 μg so that it can meet the needs of more than double that needed for library construction. A metagenomic analysis may require 50–2000 L of raw or treated water, depending on the difference in microbial concentrations in water (Djikeng et al., 2009; Rosario et al., 2009; Chao et al., 2013; Shi et al., 2013; Zhang et al., 2017). Although the biofilm phase has a much higher density of cells than the water phase in DWDS, cell collection is not easy. The main reason is the difficulty in sampling the biofilm from an actual DWDS.

Many researchers used ultrasound to transfer cells to water. Although the applications of ultrasound have been demonstrated, there is limited information available to guide selection of appropriate or optimal ultrasonic parameters. The parameters of the ultrasound, including ultrasonic power, ultrasonic duration, and the number of ultrasound treatments (USTs) chosen in previous studies varied in every aspect, such as ultrasonic power ranges from 2 W to 500 W, ultrasonic duration from 0.5 min to 20 min, and the numbers of USTs from 1 to 5 times (Table S1 in the electronic supplementary materials). Extracellular polymeric substances (EPS) promote microbe clustering and attachment to the medium, such as sediment and pipe scales. Because of the strong bonds between medium and bacteria, external disturbance might result in severe cell damage (Liu et al., 2010). At higher ultrasonic energy levels, the viability of cells can evidently drop, and cells may rupture and release DNA rapidly (Guzmán et al., 2001; Keyhani et al., 2001; Feril Jr et al., 2004), leading to failure of cell collection in the subsequent process. In contrast, at a lower ultrasonic energy level, the attached cells cannot be disintegrated from the bacteria cluster and transferred into water. Nnadozie et al. (2018) barely collected bacteria from woodchip with UST at the frequency of 40 kHz and the power of 200 W for 5 min, while after increasing the ultrasonic duration to 15 min, more cells were obtained but the quality of DNA was also affected. Williams et al. (2009) found that more than 90% cells from nontuberculous mycobacteria biofilm can be obtained with three 0.5-min USTs at the frequency of 42 kHz. Magic-Knezev and van der Kooij (2004) found that higher ultrasonic energy transferred microbes from granular activated carbon (used in water treatment) more efficiently than lower energy, and more than 90% cells can be transferred after 8 times at higher energy UST. Liu et al. (2014) found that 80%–90% of DWDS biofilm samples can be obtained after three USTs (42 kHz, 2 min) based on adenosine triphosphate (ATP) yield. However, ATP does not reflect the living state of microbes, and thus their USTs cannot guarantee microbial activity. From the literature presented above, it can be inferred that different ultrasonic parameters lead to different removal efficiencies, which will further affect the representativeness of results. The optimal ultrasonic parameters should make as many cells as possible transfer and maximize their activity. Therefore, the study of optimal ultrasonic procedure is one of the primary issues in research on DWDS biofilm.

In this study, the effects of three ultrasonic parameters, i.e. power, duration, and number of USTs on the selected monoculture bacterial biofilm (*Pelomonas sp.* biofilm) were studied; then the optimal values of each ultrasonic parameter were initially determined. Based on those values, three levels of each ultrasonic parameter were selected, an orthogonal experiment was conducted to study further on the drinking water (DW) biofilm, and finally the optimal ultrasonic parameters for the effective separation of biofilm cells in DWDS were determined. Furthermore, the effect of the optimal UST was compared with that without UST to verify the effectiveness and reliability of the present method. The results of this study aimed to provide reliable and useful data for further genotype-based studies of DWDS biofilm, so that the extracted DNA is representative and comparable across different studies.

2 Materials and methods

2.1 Monoculture bacterial biofilm

Due to the large number of replicates in heterotrophic plate count (HPC) detection, this study used a primary screening test of a culturable bacterium for

the ultrasonic parameters, a bacterium which is convenient, reliable, and economical. Fifty microliters of drinking water from the laboratory tap was spread on R2A agar medium. After incubating at 25 °C for 7 d, a colony, which was dominant on the R2A plate, was selected and transferred into 1 mL sterilized R2A liquid medium (tryptone 0.25 g, acid hydrolysis casein 0.5 g, yeast extract powder 0.5 g, soluble starch 0.5 g, KH_2PO_4 0.3 g, $MgSO_4$ 0.1 g, sodium pyruvate 0.3 g, peptone 0.25 g, glucose 0.5 g/L, and pH 7.2±0.2 at 25 °C). A stock solution of the culturable bacterium was prepared after the colony became a suspension. The selected bacterium was classified as *Pelomonas sp.* from the phylum Proteobacteria in NCBI-BLAST results. Raw data for this project were submitted to NCBI Sequence Read Archive under the project accession number PRJNA515582.

Biofilm was grown on high-density polyethylene (HDPE) coupons (surface area 21.6 cm^2) under a shear stress of 0.8 Pa at 25 °C in a reactor. An appropriate volume of the stock solution was added to 650 mL sterilized R2A liquid medium so that the final concentration of bacteria was 1.00×10^5 CFU/mL. A fed-batch operation method was adopted to form the biofilm, and the R2A liquid medium was replaced every other day.

2.2 Sampling

The sampling procedures of biofilm on coupon were: (1) the coupons were removed from the reactor with sterilized tweezers; (2) the loose bacteria were washed off 3 times gently with 1 mL sterilized phosphate buffer solution (PBS) (2 mmol/L Na_3PO_4, 4 mmol/L $NaH_2PO_4{\cdot}2H_2O$, 9 mmol/L NaCl, and 1 mmol/L KCl). For the control, the coupons were then swabbed 5 times on each surface. The cotton heads were placed in 10 mL sterilized PBS after swabbing.

The sampling procedures of biofilm in an actual DWDS were: (1) Pipe sections of 50–100 cm with different diameters and ages were removed from a test platform built in an actual DWDS in eastern China. (2) The pipe inclusion, including biofilm and sediment, was collected by swabbing the inner pipe wall with a sterile brush while continuously washing the pipe wall with 250 mL of sterile water (Liu et al., 2017). (3) The samples were transported to the laboratory at 4 °C within 4 h.

2.3 Ultrasonic parameters

An ultrasonic instrument (Biosafer 650-91, Biosafer, China) was used to transfer cells from cotton into the PBS. The ultrasonic frequency was 20 kHz, and ice bags were used to avoid overheating.

To study the optimal number of USTs, the 2-, 3-, 5-, 7-, 8-, and 9-d biofilms were sampled and treated at 6.5 W for 2 min (12 cycles of 5-s ultrasound followed by 5-s rest). Then the cotton heads were taken out and placed into 10 mL of fresh PBS. This can minimize the ultrasonic damage to the detached cells. After standing for 20 min, the cotton heads of the 2-, 3-, 5-, 7-, 8-, and 9-d biofilms were repeat-ultrasound treated 10, 10, 10, 16, 20, and 20 times, respectively. To study the optimal ultrasonic power, the 7-d biofilm was sampled and treated at 6.5, 13, 39, 78, 97.5, and 130 W for 2 min, and the number of USTs was 10. To study the optimal ultrasonic duration, the 7-d biofilm was sampled and treated at 39 W for 1, 2, 3, 4, 5, and 7 min, and the number of USTs was 10.

For biofilm in an actual DWDS, a sonication probe was placed 1 cm below the surface of the 250 mL mixture, and the sample was treated according to the optimal parameters resulting from the orthogonal experiments. After the first 20 min of standing, the supernatant was filtered with a 0.22-μm membrane so that the detached bacteria can be collected without being sonicated again, and fresh sterile water was added again. The above processes were repeated 14 times.

2.4 Orthogonal experiments design

The DW biofilm was used in an orthogonal experiment. The DW biofilm was formed on the HDPE coupons (surface area 21.6 cm^2) under a shear stress of 0.8 Pa in a reactor containing 650 mL mixture of drinking water and sterilized R2A liquid medium (1:5 in volume). The biofilm was cultivated at 25 °C for 7 d. The broth was replaced by a fresh 650 mL mixture of drinking water and sterilized R2A liquid medium every other day. HPC, total cell count (TCC), and DNA concentrations were used to select the optimal ultrasonic parameters of the DW biofilm.

Orthogonal experiments were designed by SPSS Statistics (version 20.0). After the experiment of monoculture bacterial biofilm, three levels of each parameter were selected for an orthogonal analysis

with the DW biofilm samples. The optimal ultrasonic parameters for the monoculture bacterial biofilm are the middle level, and the nearby conditions will be the lower or higher level in the orthogonal experiments. Range analysis and variance analysis, which can determine the optimal level and significance of each parameter, were conducted after the experiment. Nine trials were performed, and each trial had three replications.

2.5 DNA extraction and DNA quality

All samples were filtered by a 0.22 μm membrane (mixed cellulose, Xingya Manufacture, Shanghai, China). Then the total DNA was extracted from the sample using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) (Hwang et al., 2012). The brief protocol is as follows: after pretreatment, samples were added to a lysing matrix tube, treated with lysis buffer, and subjected to bead beating for 40 s at a maximum velocity of vortexing. DNA was bound to a silica matrix, washed and eluted in 50-μL DNase/pyrogen-free water. The extracted DNA was stored at −20 °C until further use.

To determine the DNA concentration, the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and Qubit quantitation platform (Invitrogen/Life Technologies, USA) were compared. The Nanodrop 2000 spectrophotometer is based on the principle that a nucleic acid bond absorbs ultraviolet light, while Qubit is based on the detection of target-specific fluorescence. The quality of the extracted DNA was evaluated by observing the size of the extracted DNA fragments via agarose (1.5%, weight in volume) gel electrophoresis with a DNA ladder (Solarbio Life Sciences, China) as a size standard.

2.6 HPC and quantitative polymerase chain reaction (PCR)-based TCC

HPC was conducted using the spread plate method. Ten-fold dilutions of the samples were prepared with sterilized water, and 50 μL of appropriate dilutions were spread directly onto the R2A medium. Triplicated plates were incubated at 25 °C for 7 d before enumeration of the colonies.

Quantitative PCR (qPCR) was used to quantify the TCC of the samples. Amplifications were performed in a CFX96 TouchTM Real-Time PCR Detection System (Biorad, USA). The bacterial primers 338F (ACTCCTACGGGAGGCAGCAG) and 518R (ATTACCGCGGCTGCTGG) were used to amplify a 180 bp portion of the 16S rRNA gene. Each 20 μL of reaction mix contained 10 μL of 2×SYBR Green I (Takara, Japan), 0.4 μL of forward primer (10 mmol/L), 0.4 μL of reverse primer (10 mmol/L), 8.2 μL of diethyl pyrocarbonate (DEPC)-treated water, and 1 μL of DNA template. Standard curves were generated in every qPCR run using serial dilutions of purified PCR product of near-full-length Escherichia coli 16S rRNA gene. Thermal cycling was performed using an initial denaturing step at 96 °C for 3 min followed by 40 cycles at 96 °C for 30 s, annealing at 55 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 5 min.

2.7 Data analysis

In this study, Lg HPC and Lg TCC were the base 10 logarithms of HPC (CFU/mL) and TCC (copies), respectively. Parametric one-way analysis of variance (ANOVA) was performed to compare the significance of the data. Statistical significance was set up at $p<0.05$. The increase rate reflects the proportion of obtained bacteria after each ultrasound. It was calculated by

$$\text{Increase rate}(i)=\left(\text{HPC}(i)\Big/\sum_{k=1}^{i-1}\text{HPC}(k)\right)\times 100\%, \quad (1)$$

where HPC(i) represents the HPC obtained from the ith UST, $1<i\leq n$, and n is the total number of USTs. The cumulative percentage of obtained HPC after each UST was calculated by Eq. (2), where $1\leq i\leq n$.

$$\text{Cumulative percentage}(i)=\left(\sum_{k=1}^{i}\text{HPC}(k)\Big/\sum_{k=1}^{n}\text{HPC}(k)\right)\times 100\%. \quad (2)$$

3 Results

3.1 Ultrasound experiment with monoculture bacterial biofilm

3.1.1 Number of USTs

The UST was performed 10, 10, 10, 16, 20, and 20 times on the 2-, 3-, 5-, 7-, 8-, and 9-d biofilms,

respectively. As shown in Fig. 1, obtained HPC increased with the number of USTs, and 40%–80% of cells were obtained from the first ultrasound. The increase rate reflects the proportion of obtained bacteria after each ultrasound. The increase rates were high for the first 4 ultrasounds, but by the 5th time, the rate had clearly decreased (Table S2). Based on the results, 2% was set as the lower limit boundary of the increase rate to select the suitable number of USTs. That is, when the rate is less than 2%, further UST does not make much sense for collecting more bacteria. The increase rates of all biofilm samples at the 11th ultrasound were less than 2%. Therefore, 10 was considered as the optimal number of USTs for monoculture bacterial biofilm.

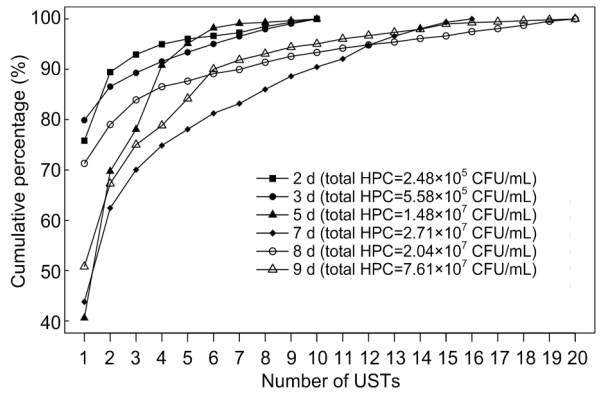

Fig. 1 Cumulative percentage of obtained bacteria in monoculture biofilm samples under different numbers of USTs

3.1.2 Ultrasonic power

As shown in Fig. 2, after conducting the UST for 10 times, the most HPC were obtained at 39 W, while the least HPC were obtained at 78 W. The lower power (6.5 W, 13 W, and 39 W) shows a higher increase rate for the second (p=0.07) and third (p=0.06) ultrasounds than the higher power (78 W, 97.5 W, and 130 W), and the power becomes equivalent at the subsequent ultrasounds. This result indicates that the different power levels have more of an effect for the first three ultrasounds than for the subsequent ultrasounds. Therefore, the power of 39 W was considered as the optimal ultrasonic power for the monoculture bacterial biofilm.

3.1.3 Ultrasonic duration

Ultrasonic duration is also an important parameter. A long period of ultrasound is likely to increase the rate of microbial rupture. In previous studies (Table S1), 1 min and 2 min were the most common ultrasonic durations. Thus, 1 min to 7 min was selected in this study.

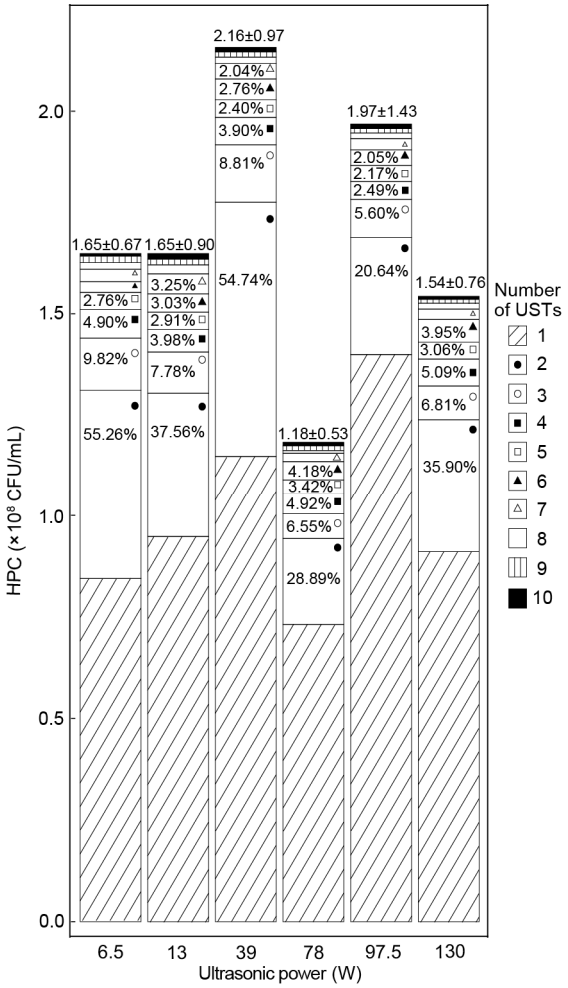

Fig. 2 HPC and increasing rates of monoculture bacterial biofilm samples under different ultrasonic powers

Percentage represents increase rate under specific UST. Increase rates of less than 2% are not shown. Number above bar represents total HPC obtained after 10 USTs, average± standard deviation

The effect of ultrasonic duration on HPC has an obvious trend: a longer duration causes more damage to active cells, as shown in Fig. 3. After treatment for 10 times, 1-min and 2-min ultrasounds can transfer

the most HPC, whereas the HPC of the 7-min ultrasound decreased significantly with negligible increase in HPC after the third treatment. Average 5.67×10^7 CFU/mL can be obtained after 1 to 2-min UST, 38% of the HPC decreases after 3 to 5-min UST, and 70% of the HPC decreases after 7-min UST. The HPC of 1-min samples is less than that of the 2-min samples for the first four ultrasounds, but the HPC of the 1-min samples reach the HPC of the 2-min samples at the 5th ultrasound. For the first five ultrasounds, the increase rate of the 1-min ultrasound is larger than that of the 2-min ultrasound, but the increase rates of the subsequent ultrasounds were similar, which indicates that the removal ability of the 2-min ultrasound to transfer cells is stronger than that of the 1-min ultrasound. Therefore, 2 min was considered as the optimal ultrasonic duration for the monoculture bacterial biofilm.

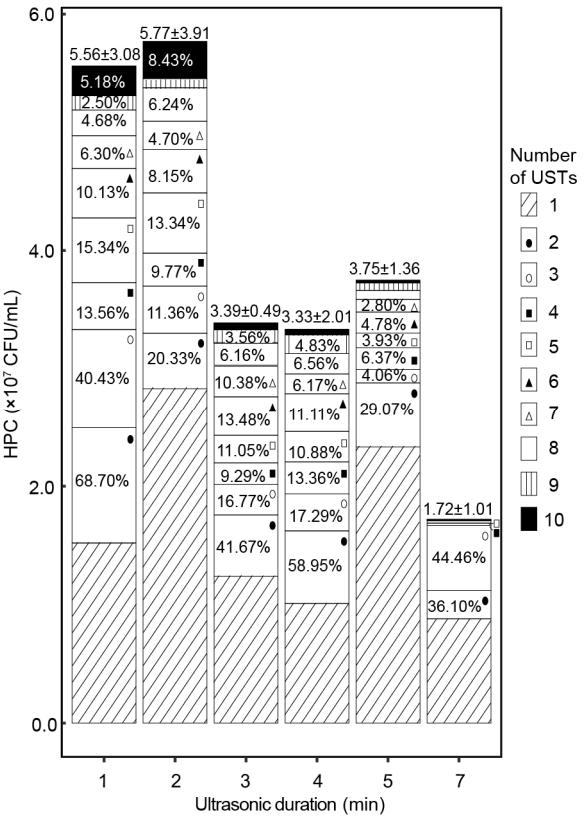

Fig. 3 HPC and increase rates of monoculture bacterial biofilm samples under different ultrasonic durations
Percentage represents the increase rate under specific UST. Increase rates of less than 2% are not shown. Number above bar represents total HPC obtained after 10 USTs, average± standard deviation

3.2 Orthogonal experiment with DWDS biofilm

As a branch of the factorial experimental design, the orthogonal design of an experiment is used to study the effect of multiple factors simultaneously in a single set of experiments with many fewer experiment units and to identify the optimal level of the factors. Orthogonal experiments can also determine the significant factors through variance analysis. In this study, three levels of each factor were selected in the orthogonal experiments to identify the optimal ultrasonic parameters for DWDS biofilm.

The results of the HPC, TCC, and DNA concentration of each trial are shown in Table 1. The range analysis and variance analysis of HPC, TCC, and DNA concentration are listed in Tables S3–S5. A factor would exert a stronger influence on the index when the *R*-value was larger in the range analysis. The comparison of the *R*-values of HPC (Table S3a) gives the result that ultrasonic duration>number of USTs>ultrasonic power. The contrast of the *R*-values of TCC and DNA concentrations (Tables S4a and S5a) gives the result of number of USTs>ultrasonic duration>ultrasonic power. For DWDS biofilm, HPC can only reflect the number of heterotrophic rather than live bacteria, so that TCC and DNA concentration are better for revealing the situation of the DWDS biofilm. Thus, the number of USTs is the main influencing factor, followed by ultrasonic duration and power.

In the present study, the aim is to select the appropriate ultrasonic parameters to ensure that HPC, TCC, and DNA concentration are as high as possible. Therefore, the highest level of each curve is the optimal level in Fig. 4, which was made based on range analysis (Tables S3a, S4a, and S5a). For HPC, the range analysis shows that 10 USTs at 13 W for 1 min is optimal (Fig. 4a). For TCC, it shows that 15 USTs at 13 W for 1 min is optimal (Fig. 4b), and the number of USTs shows a significant effect through the variance analysis ($F>F_{0.05}$, Table S4b). For DNA concentration, the range analysis also indicates that 15 USTs at 13 W for 1 min is optimal (Fig. 4c), and the number of USTs also shows a significant effect through the variance analysis ($F>F_{0.05}$, Table S5b). The optimal parameters obtained from the orthogonal experiments are different from those obtained from the monoculture biofilm. The variance analyses of the

orthogonal results show that the number of USTs has a significant effect on TCC and DNA concentration (p<0.05), so 15 is considered as an optimal number of USTs for DWDS biofilm. The range analysis of three factors in the orthogonal experiments shows that a 13-W and 1-min ultrasound can transfer the most cells. Therefore, an ultrasound at 13 W for 1 min is considered as an optimal condition for the DWDS biofilm.

Table 1 Results of orthogonal experiment

Trial	Power (W)-duration (min)-number of USTs	Lg HPC	Lg TCC	DNA concentration (ng/μL)
1	13-1-5	7.92±0.10	10.27±0.37	107.47±20.08
2	13-2-10	8.03±0.11	10.61±0.48	131.67±52.25
3	13-3-15	7.91±0.12	11.05±0.99	152.33±43.66
4	39-1-15	8.01±0.10	11.12±0.79	147.00±7.21
5	39-2-5	7.98±0.21	10.19±0.26	97.33±18.41
6	39-3-10	7.79±0.06	10.60±0.46	108.20±57.29
7	78-1-10	8.08±0.10	10.65±0.47	155.87±62.29
8	78-2-15	7.81±0.25	11.00±0.65	138.67±20.82
9	78-3-5	7.90±0.22	10.24±0.30	85.93±23.54

3.3 Verification with biofilm in an actual DWDS

The yield DNA with an optimal UST and without UST is shown in Table 2. The portions of sediments were determined by total solid and volatile solid in the authors' previous study: averagely 86.82%, 88.82%, and 89.43% of the collection were environmental substances from the cement-lined ductile iron cast pipe, steel pipe, and HDPE pipe, respectively (Ren, 2016). Without UST, the trend of extracted DNA did not match that of HPC. Nearly no DNA was obtained from two of the samples, though they have high HPC in biofilm. However, with optimal UST, DNA can be extracted from all samples, and the obtained amount of DNA increased at least 4.78 times and even up to 26.8 times. All samples reached the requirement of 0.1 μg for the metagenomic library. Most of the samples reached the requirement of 2 μg, which indicated that water quality, pipelining, and sediments do affect DNA extraction. The optimal ultrasonic method has a positive effect on increasing the amount of DNA in DWDS biofilm. The lengths of extracted DNA were detected by agarose gel electrophoresis. Compared to the samples without UST, the samples from actual DWDS with optimal UST have the same quality (i.e. DNA fragment size). Each sample has similar brightness of bright band, and the extracted DNA was mainly 15 000 bp, as shown in Fig. 5. This indicated that the optimal UST will not lose DNA quality.

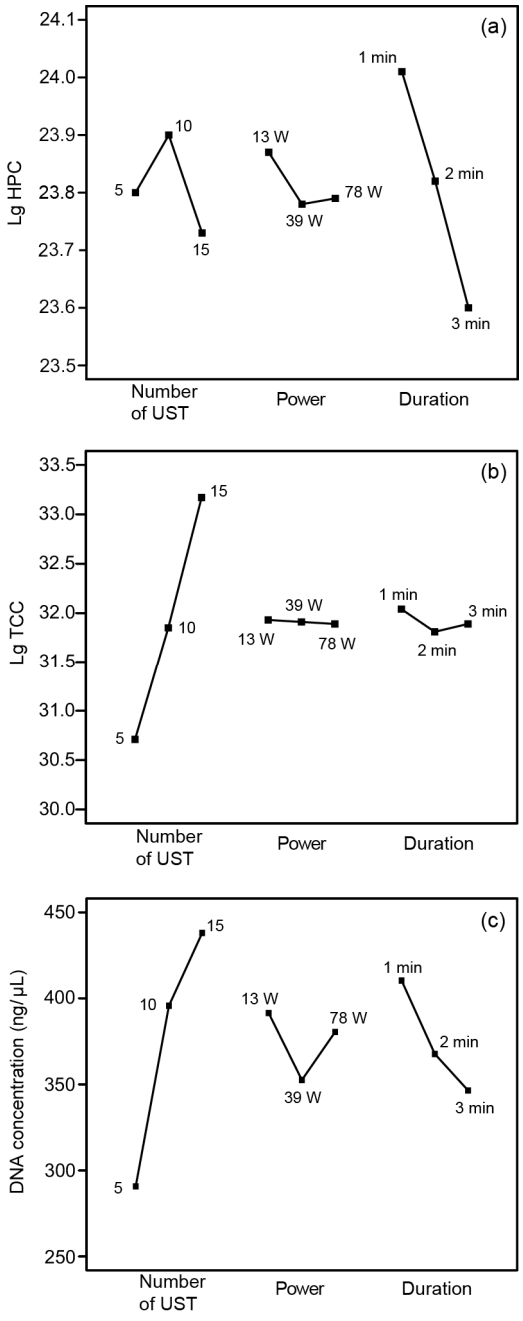

Fig. 4 Effects of number of USTs, power, and duration on HPC (a), TCC (b), and DNA concentration (c)

Table 2 DNA extraction in samples from an actual DWDS with optimal UST and without UST

Pipe material	Pipe age (a)	Sampling area (cm^2)	With optimal UST		Without UST	
			HPC (CFU/cm^2)	DNA amount (μg)	HPC (CFU/cm^2)	DNA amount (μg)
Cement-lined ductile iron cast	3	1256	4.01×10^4	0.33	8.89×10^3	0
Cement-lined ductile iron cast	7–8	6280	3.22×10^4	2.15	2.32×10^4	0
Cement-lined ductile iron cast	7–8	3140	1.39×10^4	0.46	3.28×10^2	0.09
Steel	1–2	1256	7.83×10^4	3.20	4.19×10^3	0.67
HDPE	1	1256	5.84×10^3	2.01	3.18×10^3	0.08
HDPE	7–8	6280	3.84×10^4	1.34	1.61×10^5	0.05

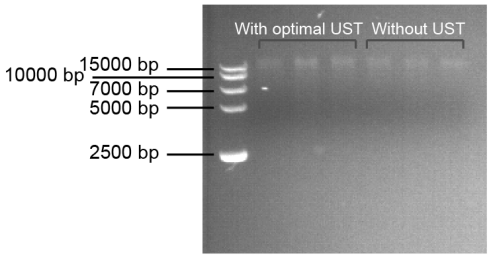

Fig. 5 Agarose gel electrophoresis of DNA isolated from samples from cement-lined ductile iron cast pipe (sampling area 60 cm^2) with optimal UST and samples from steel pipe (sampling area 60 cm^2) without UST

4 Discussion

The effect of different ultrasonic parameters on biofilm detachment was compared in this study.

The aim is to find appropriate ultrasonic parameters for biofilm in DWDS, so that as many live cells as possible can be collected from the attached medium for further analysis. This makes results more representative and comparable. There is not only intercellular DNA but also extracellular DNA existing in biofilm. However, extracellular DNA in EPS has been reported as being detected at very low concentration by staining methods (Fish et al., 2017). In addition, it has been reported that extracellular DNA occur in larger amounts in waste-water biofilms than in biofilm from other origins (Flemming and Wingender, 2010), but as was concluded by Liu and Fang (2002), only 1.1%–1.2% of DNA in sludge samples were detected as extracellular DNA. On the other hand, extracellular DNA is the residual material from lysed cells, which may not be collected during filtration with a 0.22 μm membrane. Therefore, the extracted DNA is hereafter referred to as intercellular DNA. Since the selected bacterium was culturable, HPC can be referenced as the total counts of bacteria with normal activity as well as the DNA yield indirectly. The selected bacterium, *Pelomonas*, is a genus of Gram-negative bacteria from the phylum Proteobacteria. Thus, we designed the culturable monoculture experiment to initially narrow down the range of ultrasonic parameters.

In previous studies, one to five USTs were conducted to detach cells (Table S1), which, according to the present results, may result in a loss of up to 60% of bacteria. However, microbial activity is negatively affected by continuous UST. Liu et al. (2014) found that the relative ATP yield showed an increase in the first three USTs but decreased with additional treatments. Magic-Knezev and van der Kooij (2004) also found the increase-then-decrease trend of ATP concentration and HPC after USTs. It is believed that the increasing ATP concentration and HPC were attributed to a disintegration of clusters of bacteria during the ultrasound, while decreasing HPC was a result of the action of nucleotide released on individual bacterium caused by further ultrasound. To reduce the damage of ultrasound, suspension bacteria should be collected after each UST to avoid secondary shock. As opposed to the previous studies, protection against suspended bacteria was taken into account in the present study. The results of the monoculture provide practical guidance, that is, 70% of bacteria can be obtained after conducting an ultrasound 4 times, 90% of bacteria can be obtained after 10 times, and 95% of bacteria can be obtained after 15 times (Fig. 1). The more bacteria we collect, the more representative the sample is. In the orthogonal test, more TCC and DNA yield but less HPC were obtained by treating 15 times than 10 times. Previous study also showed that culturable cells increase after

first few USTs but obviously decrease in further treatment because of cell eruption (Magic-Knezev and van der Kooij, 2004). Based on the present results, the increase in unculturable cells is more than the offset by the lost culturable cells. There are two types of viable but non culturable (VBNC) bacteria. The first type is caused by the fact that the conditions for growth in artificial media have not yet been selected (Dorofeev et al., 2014). The second type is bacteria incapable of forming a colony without a preliminary stage of resuscitation. As far as we know, there is no evidence about ultrasound inducing culturable bacteria into VBNC status. The situation in the present study may be caused by the differences in the bacterial growth phase. A culturable bacterium, *Enterobacter aerogenes*, was found to be sensitive to ultrasound in its exponential growth phase (Gao et al., 2014). However, not all culturable bacteria have the same property. *Bacillus subtilis* and *Staphylococcus epidermidis* were not affected by the growth phase (Gao et al., 2014). The sampling swabs used in this study were transferred to a new tube containing PBS for the next UST, which allows the remaining bacteria to access more nutrients. After a period of adaption, some culturable bacteria that may get into the exponential growth state after 10 USTs, become sensitive to ultrasound.

In previous studies, ultrasonic power varied from 2 W to 500 W (Table S1). Magic-Knezev and van der Kooij (2004) had ultrasonically treated granular activated carbon filters 10 times at 20 kHz and found that higher removal was achieved at 40 W than 20 W. In this study, the most monoculture bacteria can be obtained at 39 W rather than another power, but there was no linear relationship between ultrasonic power and total HPC. The difference between lower and higher power can only be found in the increase rate of HPC. Lower power showed higher increase rate than higher power at the second and third treatments. The orthogonal test results reflected the differences between the monoculture biofilm and actual biofilm. For DWDS biofilm, the range analysis of the orthogonal test showed that more HPC, TCC, and DNA yield were obtained at 13 W than 39 W.

The effect of duration on obtained cells was significant. Longer duration was more harmful to cell activity (Fig. 3). This result is consistent with a previous study (Saccani et al., 2014). Long duration brings continuous ultrasound shock to the detached and attached cells, and even intercellular DNA. To avoid continuous ultrasound shock, it is necessary for a medium with attached cells to recover for 20 min after each ultrasound (Williams et al., 2011). Ultrasonic degradation of DNA in solution occurs by breaking hydrogen bonds and by single-strand and double-strand ruptures of the DNA helix (Elsner and Lindblad, 1989). Elsner and Lindblad (1989) also found that ultrasonic duration significantly shortened DNA fragment length to a limit of 100–500 bp. Compared to the samples without UST, the samples from actual DWDS with optimal UST had the same quality (e.g. DNA fragment size), which was mainly 15000 bp in the present study (Fig. 5). This indicated that the optimal UST will not lose DNA quality. DNA in cells may be protected by sediments and other attached media, because they have a high absorption coefficient of sound (Oelze et al., 2002).

Finally, two DNA quantification methods, namely Nanodrop spectrophotometer and Qubit quantitation platform, were compared. The Nanodrop spectrophotometer is convenient and a commonly used piece of equipment in laboratories, and is based on the principle that nucleic acid bond absorbs ultraviolet light. The Qubit quantitation platform is a fluorescent-based quantitation. DNA concentrations of five 10-fold dilutions are shown in Fig. 6. The result shows that the DNA concentration measured by Qubit is more accurate than by Nanodrop. A lower DNA concentration results in a more significant difference between the two methods, with a maximum deviation of 11.3 ng/μL. Furthermore, the DNA concentration measured by Qubit shows a 10-fold change with 10-fold dilutions, whereas the DNA concentration measured by Nanodrop does not show the corresponding trend in 100-fold and higher dilutions. DNA concentration will be overestimated by ultraviolet spectroscopy, which results from the existence of RNA, protein, free nucleotides, and so on. This error cannot be ignored for samples with low DNA concentration. Fluorescence quantification uses molecular probes, which can specifically bind to DNA and emit light. Therefore, Qubit is more accurate and particularly suitable for samples with low DNA concentration, such as DWDS biofilm samples. These findings were also validated by Hwang et al. (2012).

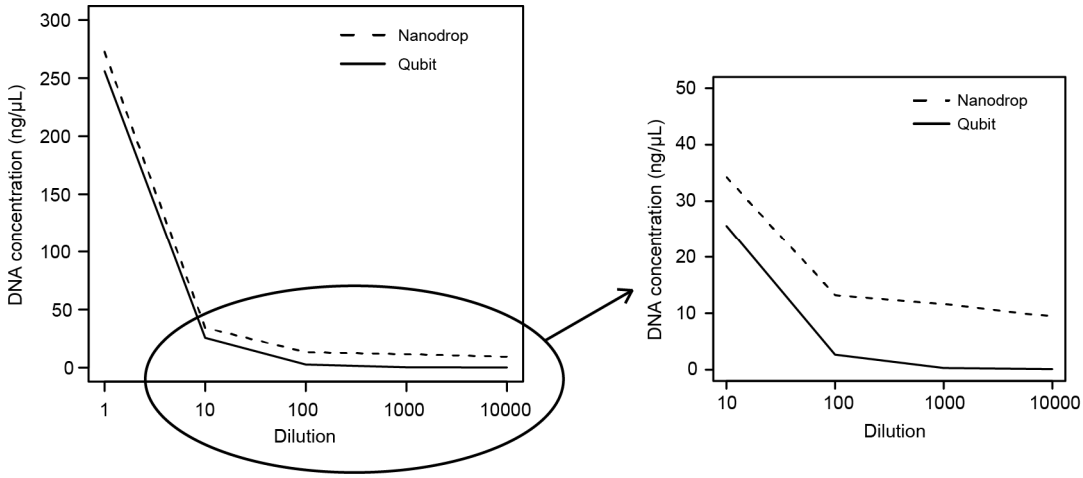

Fig. 6 DNA concentrations of 10-fold dilutions measured by Nanodrop 2000 spectrophotometer and Qubit quantitation platform

"1" represents the sample without dilution, and the bacteria concentration is 1×10^8 CFU/mL

5 Conclusions

The following major experimental observations and conclusions can be drawn from this study. At the ultrasonic frequency of 20 kHz, lower power (6.5, 13, and 39 W) can better protect the microbial activity of a monoculture bacterial biofilm, and can transfer as many microbes as possible from the medium by multiple times of UST. For DWDS biofilm, the number of USTs is the main factor affecting microbial transfer, followed by the ultrasound duration and ultrasound power. The optimal UST for cells collection in an actual DWDS is as follow: pipe inclusions (such as sediments and pipe scales) from actual DWDS should be ultrasonically treated at 13 W for 1 min, and then the sampling medium or pipe inclusions should be transferred into fresh PBS and left to stand still for 20 min with the ultrasound, and then repeated 14 times. Compared to the treatment without ultrasound, the present optimal UST can increase DNA yield by at least 4.78 times without reducing DNA quality. This study has better solved the problem of DNA collection for molecular biotechnology in DWDS biofilm.

Contributors

Hong-xi PENG and Jing-qing LIU designed the research. Hong-xi PENG, Yi-fu ZHANG, and Ruo-wei WANG performed the experiments. Hong-xi PENG processed the corresponding data and wrote the first draft of the manuscript. Yu SHAO, David Z. ZHU, Huan-yu CHEN, and Jing-qing LIU helped to organize the manuscript. Hong-xi PENG revised and edited the final version.

Conflict of interest

Hong-xi PENG, Yu SHAO, Yi-fu ZHANG, Ruo-wei WANG, David Z. ZHU, Huan-yu CHEN, and Jing-qing LIU declare that they have no conflict of interest.

References

Chao YQ, Ma LP, Yang Y, et al., 2013. Metagenomic analysis reveals significant changes of microbial compositions and protective functions during drinking water treatment. *Scientific Reports*, 3:3550. https://doi.org/10.1038/srep03550

Djikeng A, Kuzmickas R, Anderson NG, et al., 2009. Metagenomic analysis of RNA viruses in a fresh water lake. *PLoS One*, 4(9):e7264. https://doi.org/10.1371/journal.pone.0007264

Dorofeev AG, Grigor'eva NV, Kozlov MN, et al., 2014. Approaches to cultivation of "nonculturable" bacteria: cyclic cultures. *Microbiology*, 83(5):450-461. https://doi.org/10.1134/S0026261714050087

Douterelo I, Sharpe RL, Boxall JB, 2013. Influence of hydraulic regimes on bacterial community structure and composition in an experimental drinking water distribution system. *Water Research*, 47(2):503-516. https://doi.org/10.1016/j.watres.2012.09.053

Elsner HI, Lindblad EB, 1989. Ultrasonic degradation of DNA. *DNA*, 8(10):697-701. https://doi.org/10.1089/dna.1989.8.697

Feril Jr JB, Kondo T, Takaya K, et al., 2004. Enhanced

ultrasound-induced apoptosis and cell lysis by a hypotonic medium. *International Journal of Radiation Biology*, 80(2):165-175. https://doi.org/10.1080/09553000310001654684

Fish K, Osborn AM, Boxall JB, 2017. Biofilm structures (EPS and bacterial communities) in drinking water distribution systems are conditioned by hydraulics and influence discolouration. *Science of the Total Environment*, 593-594:571-580. https://doi.org/10.1016/j.scitotenv.2017.03.176

Flemming HC, Wingender J, 2010. The biofilm matrix. *Nature Reviews Microbiology*, 8(9):623-633. https://doi.org/10.1038/nrmicro2415

Gao SP, Lewis GD, Ashokkumar M, et al., 2014. Inactivation of microorganisms by low-frequency high-power ultrasound: 1. Effect of growth phase and capsule properties of the bacteria. *Ultrasonics Sonochemistry*, 21(1):446-453. https://doi.org/10.1016/j.ultsonch.2013.06.006

Gomes IB, Simões M, Simões LC, 2014. An overview on the reactors to study drinking water biofilms. *Water Research*, 62:63-87. https://doi.org/10.1016/j.watres.2014.05.039

Guzmán HR, Nguyen DX, Khan S, et al., 2001. Ultrasound-mediated disruption of cell membranes. I. Quantification of molecular uptake and cell viability. *The Journal of the Acoustical Society of America*, 110(1):588-596. https://doi.org/10.1121/1.1376131

Hwang C, Ling FQ, Andersen GL, et al., 2012. Evaluation of methods for the extraction of DNA from drinking water distribution system biofilms. *Microbes and Environments*, 27(1):9-18. https://doi.org/10.1264/jsme2.ME11132

Ji P, Rhoads WJ, Edwards MA, et al., 2017. Impact of water heater temperature setting and water use frequency on the building plumbing microbiome. *The ISME Journal*, 11(6):1318-1330. https://doi.org/10.1038/ismej.2017.14

Keyhani K, Guzmán HR, Parsons A, et al., 2001. Intracellular drug delivery using low-frequency ultrasound: quantification of molecular uptake and cell viability. *Pharmaceutical Research*, 18(11):1514-1520. https://doi.org/10.1023/A:1013066027759

Lehtola MJ, Laxander M, Miettinen IT, et al., 2006. The effects of changing water flow velocity on the formation of biofilms and water quality in pilot distribution system consisting of copper or polyethylene pipes. *Water Research*, 40(11):2151-2160. https://doi.org/10.1016/j.watres.2006.04.010

Liu G, Bakker GL, Li S, et al., 2014. Pyrosequencing reveals bacterial communities in unchlorinated drinking water distribution system: an integral study of bulk water, suspended solids, loose deposits, and pipe wall biofilm. *Environmental Science & Technology*, 48(10):5467-5476. https://doi.org/10.1021/es5009467

Liu H, Fang HHP, 2002. Extraction of extracellular polymeric substances (EPS) of sludges. *Journal of Biotechnology*, 95(3):249-256. https://doi.org/10.1016/S0168-1656(02)00025-1

Liu J, Li JQ, Feng L, et al., 2010. An improved method for extracting bacteria from soil for high molecular weight DNA recovery and BAC library construction. *The Journal of Microbiology*, 48(6):728-733. https://doi.org/10.1007/s12275-010-0139-1

Liu JQ, Luo ZF, Liu K, et al., 2017. Effect of flushing on the detachment of biofilms attached to the walls of metal pipes in water distribution systems. *Journal of Zhejiang University-SCIENCE A (Applied Physics & Engineering)*, 18(4):313-328. https://doi.org/10.1631/jzus.A1600316

Magic-Knezev A, van der Kooij D, 2004. Optimisation and significance of ATP analysis for measuring active biomass in granular activated carbon filters used in water treatment. *Water Research*, 38(18):3971-3979. https://doi.org/10.1016/j.watres.2004.06.017

Nnadozie CF, Lin J, Govinden R, 2018. Optimisation of protocol for effective detachment and selective recovery of the representative bacteria for extraction of metagenomic DNA from *Eucalyptus* spp. woodchips. *Journal of Microbiological Methods*, 148:155-160. https://doi.org/10.1016/j.mimet.2018.04.009

Oelze ML, O'Brien WD, Darmody RG, 2002. Measurement of attenuation and speed of sound in soils. *Soil Science Society of America Journal*, 66(3):788-796. https://doi.org/10.2136/sssaj2002.7880

Ren HX, 2016. The Spatial-temporal Distribution and Dynamic Change of Microbial Community in Drinking Water Supply System. MS Thesis, Zhejiang University, Hangzhou, China (in Chinese).

Rosario K, Nilsson C, Lim YW, et al., 2009. Metagenomic analysis of viruses in reclaimed water. *Environmental Microbiology*, 11(11):2806-2820. https://doi.org/10.1111/j.1462-2920.2009.01964.x

Saccani G, Bernasconi M, Antonelli M, 2014. Optimization of low energy sonication treatment for granular activated carbon colonizing biomass assessment. *Environmental Technology*, 35(7):851-858. https://doi.org/10.1080/09593330.2013.853698

Shi P, Jia SY, Zhang XX, et al., 2013. Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water. *Water Research*, 47(1):111-120. https://doi.org/10.1016/j.watres.2012.09.046

Williams DL, Woodbury KL, Haymond BS, et al., 2011. A modified CDC biofilm reactor to produce mature biofilms on the surface of PEEK membranes for an in vivo animal model application. *Current Microbiology*, 62(6):1657-1663. https://doi.org/10.1007/s00284-011-9908-2

Williams MM, Yakrus MA, Arduino MJ, et al., 2009. Structural

analysis of biofilm formation by rapidly and slowly growing nontuberculous mycobacteria. *Applied and Environmental Microbiology*, 75(7):2091-2098. https://doi.org/10.1128/AEM.00166-09

Zhang Y, Oh S, Liu WT, 2017. Impact of drinking water treatment and distribution on the microbiome continuum: an ecological disturbance's perspective. *Environmental Microbiology*, 19(8):3163-3174. https://doi.org/10.1111/1462-2920.13800

Zhou LL, Zhang YJ, Li GB, 2009. Effect of pipe material and low level disinfectants on biofilm development in a simulated drinking water distribution system. *Journal of Zhejiang University-SCIENCE A*, 10(5):725-731. https://doi.org/10.1631/jzus.A0820486

Zhu ZB, Wu CG, Zhong D, et al., 2014. Effects of pipe materials on chlorine-resistant biofilm formation under long-term high chlorine level. *Applied Biochemistry and Biotechnology*, 173(6):1564-1578. https://doi.org/10.1007/s12010-014-0935-x

List of electronic supplementary materials

Table S1 Ultrasonic parameters for cell detachment in previous studies

Table S2 Increasing rate of living bacteria after every ultrasound treatment

Table S3a Range analysis on Lg HPC

Table S3b Variance analysis on Lg HPC

Table S4a Range analysis on Lg TCC

Table S4b Variance analysis on Lg TCC

Table S5a Range analysis on DNA concentration

Table S5b Variance analysis on DNA concentration

中文概要

题　目：促进饮用水供水管道生物膜脱落的超声处理参数优化

目　的：从贫营养环境（如饮用水供水管道）中获取足量的 DNA 是用分子生物学的方法研究微生物群落的重要环节。尽管如此，DNA 量不足始终是对饮用水环境样品进行宏基因组测序时最大的问题之一。为了获得尽可能多的微生物，本文采用超声处理进行微生物分离，并研究处理供水管道生物膜的最佳超声处理参数。

创新点：本研究提供了一种预处理方法，可以从实际供水管道生物膜中提取更多可靠的 DNA，可以更好地解决贫营养环境中 DNA 的收集问题。

方　法：1. 用挑选的细菌（*Pelomonas sp.*）形成的生物膜来初步研究三个超声参数的最佳水平，即功率、时间和超声处理次数；2. 基于这些值，对每个超声参数选择三个水平，并对实验室培养的饮用水生物膜进行正交试验，以进一步确定能有效分离生物膜的最佳超声参数水平；3. 使用研究所得的最优超声处理参数对从实际供水管道采集的生物膜进行处理，以验证其效果。

结　论：1. 最佳超声功率、时间和次数分别为 13 W、1 min 和 15 次，且两次超声处理之间需要 20 min 的静置期；2. 与没有进行超声处理的样品相比，研究所得的最优超声处理不会降低 DNA 质量，而且可以将提取的 DNA 量增加至少 4.78 倍。

关键词：生物膜；饮用水供水管道；DNA 量；DNA 质量；超声处理