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Evaluation and Optimization of Methods for the DNA Extraction from Drinking water

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Abstract

Tap water qualification is quite important for human health, the DNA extraction from tap water is a current challenge since the microbes inside is limited. This article aims to evaluate various DNA extraction methods and based on the different reagents or procedures and their influence to do some modifications for better extraction. DNA yield, purity and fragment size are three evaluation criteria for extracted DNA, while the reproducibility and operational convenience are also taken into evaluation for each method. According to the results, extraction methods could be recommended for different situations. It is concluded that commercial kits show better reproducibility in yield and quality aspects and more convenient in operation than phenol/chloroform based methods. The modified method which adds enzymatic and chemical lysis method into standard commercial kit protocol, shows the highest DNA yield. The phenol/chloroform based methods give the best average purity, especially for protein contaminants. Considering the fragment size, the DNA extracted from Quick-DNA HMW MagBead kit (ZYMO Research, USA) distributes widest with more HMW DNA among all methods. The results presented here suggest that that the DNA extraction method of choice for tap water samples should be the modified Quick-DNA HMW MagBead kit.

keywarods : DNA extraction, tap water

Contents

1 Introduction

1.1 Microbes in the tap water

Tap water is widely drunk directly in the Netherlands. However, the qualification of tap water are always ignored by the public, compared with treated water. The biomass from pipe wall biofilms and followed loose deposits accounts for over 98% in the drinking water distribution system (DWDS) [Liu et al., 2014]. With loose deposits from biofilms happened in the transportation through DWDS, tap water might have larger amounts of microbes and different microbial community structure with treated water.

Pathogens in the tap water are a kind of microbial contamination which is a major cause of food-borne illness [Yam & Lee, 2012]. As a result, it is necessary to detect the microbes inside the tap water. Detection of drinking water samples could help the drinking water company better manage the drinking water quality.

Mostly methods that widely used in DNA extraction have been developed for soil samples, including most commercial kits. Unlike the large quantities of biomass inside soil samples, the amount of microorganisms in the drinking water is limited with a microbial concentration range between $10^3$and 10^5 cells/ml [Solize Vosloo, 2019], which causes a big challenge to extract DNA in the drinking water samples. Besides, the harmful substances or clinic pathogens inside the tap water samples might be interfered during DNA extraction process and cause negative effect on the following sequencing or other analysis. As a result, high quality and quantity DNA extracted from tap water is necessarily needed for the detection of tap water quality.

1.2 DNA extraction and purification mechanisms

Considering the mechanisms of various methods, extraction methods could be divided into the following several groups with different lysis mechanisms or different reagents and procedures used during the extraction. It is therefore significantly useful to learn the impact of procedures or reagents biases on the final extraction output.

Enzymatic, chemical and physical treatment are three main kinds of lysis mechanisms during extraction process. Enzymatic treatment is a biological cell lysis method which applies enzymes that dissolves the cell wall. Causing bacteria in the tap water samples are the subject we focused, lysozyme is specificly applied for bacterial cell lysis in our research. Lysozyme is an antimicrobial enzyme that can hydrolyze the 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine in the peptidoglycan of bacterial cell wall [Arnheim et al., 1973]. For this reason, gram-positive bacteria can be directly exposed to lysozyme [Niwa et al., 2005]. When applied to Gram-negative bacteria, it should be supplemented with Ethylenediaminetetraacetic acid (EDTA) for higher effective on the cell wall [Salazar & Asenjo, 2007].

Lysozyme treatment is generally conducted at pH 6–7 buffer and incubated at 37°C, specific incubation time depends on the working concentration of lysozyme in different lysis buffers [Harrison, 1991]. However, this lysis method is highly impossible to lyse the cell wall completely, thus, other lysis methods like freeze-thaw technique or osmotic pressure might be followed after this lysis process to destroy the cell membrane. What is more, for bacterial cells, combination of detergents is compulsory and the high expense of the reagents for this lysis method are both its disadvantages [Shehadul Islam et al., 2017].

In the aspect of chemical lysis treatment, lysis buffer is used to break the cell membrane and release intercellular components. Chemical reagents like acids or bases could lyse the cell membrane by controlling the pH. An alkaline environment with pH 11.5-12.5 is suitable for cell lysis [Stanbury et al., 2013]. However, this method would take a quite long time for the lysis process, so it is not widely applied in extraction methods.

Besides this, detergents are another important components in the lysis buffer which could solubilize the proteins and lipids that form the bilipid cell membranes by disrupting the hydrophobic-hydrophilic interactions[Shehadul Islam et al., 2017]. Sodium dodecyl sulphate (SDS) is a strong anionic lysis agent, since its high affinity that could bind to proteins and denature them quickly. Ethylenediaminetetraacetic acid (EDTA) is an another chaotropic detergent, which could weaken the hydrophobic interactions to break the hydrogen bonding network between water molecules. Consequently, a negative effect should be given on the stability of the native state of other molecules in the solution, mainly for macro-molecules such as proteins, nucleic acids. As a result, EDTA always works as incorporation with other lysis methods, especially for soil samples [Jacobsen & Rasmussen, 1992].

Based on the mechanisms of detergents during lysis process, an extra purification step has to be considered and attached to the cell lysis protocol to remove membrane barriers [Jacobsen & Rasmussen, 1992, R. Sharma et al., 2012]. When applied to lyse bacterial cells, the cell wall has to be broken down firstly in order to give the detergents an access to the cell membrane. Thus, detergents are often used in company with lysozymes for lysing process [Shehadul Islam et al., 2017]. What is more, there are

other supplement procedures that could be helpful to chemical treatment, for example, high salt and high temperature incubation (mostly 60°C to 70°C) [Bruce et al., 1992, Kuske et al., 1998].

Physical and mechanical lysis methods are two methods which rupture cell membrane with utilization of external force. The difference between these two methods is that mechanical lysis use direct contact with cells, while physical lysis is a non-contact method [Shehadul Islam et al., 2017]. Physical methods applies various forces, including heat, pressure and sound energy, which corresponding with freeze-thaw disruption [Kuske et al., 1998, Tsai & Olson, 1991], osmotic shock [Shehadul Islam et al., 2017] and ultrasonication [Picard et al., 1992].

Osmotic shock uses the osmotic pressure caused by sudden change of the concentration of salt surrounding the cells. With concentration of extracellular solution is lower than intercellular solution, water penetrates into cells , thus makes the cells swell up and burst.

For freeze-thaw disruption, formation of ice on the cell membrane is the main reason that helps rupture it. Besides, high temperature do not only thaw the samples, but also denaturize the membrane proteins to rupture the cell membrane [Shehadul Islam et al., 2017]. Osmotic shock method was found as the highest yield when compared with ultrasonication and even some mechanical methods [Byreddy et al., 2015]. Freeze-thaw technique is the one used more widely since its easy operation. However, this method is time-consuming and not suitable for extraction of components sensitive to temperature. Modifications based on this method like lysozyme treatment prior to freeze-thaw lysis could improve the efficiency of cell lysis [Zhu et al., 2006].

Mechanical lysis method widely uses beat mill, and homogenization or vortex are two kinds machines used for bead mill. Bead mill homogenization was convinced that yields more DNA than freeze-thaw techniques [Kuske et al., 1998, Leff et al., 1995, Miller et al., 1999].Since the significantly advantage of this method, bead mill is commonly applied for cell lysis, especially for commercial kits. However, DNA shearing is the main drawback of this high yields method [Leff et al., 1995].In reality, different kinds of lysis methods are always incorporation with others to improve the extraction efficiency.

For better purity of extracted DNA, purification is also an important process after cell lysis. Membrane lipids, protein and other cellular contamination are three main things that need to be removed in basic extraction methods. Several methods could be applied for purification with various mechanisms.

The mostly widely used conventional one is phenol/chloroform method. Centrifugation is used for the removal of cell debris following cell lysis process. Protease always works for protein denaturation or digestion, then the denatured protein could be precipitated by organic solvents and removed by centrifugation. One of the organic solvents that widely used for the precipitation is 24:1 (v:v) phenol and chloroform mixture and this purification method is adopted by direct lysis methods [Hwang et al., 2009, Zhou et al., 1996]. Purified DNA could be extracted by precipitation with ethanol or isopropanol. 70% ethanol should be used as the wash solvent in this method to remove residual organic contents. The dangerous organic solvents used in this method is a significant drawback and the possible residual might have a negative effect on the downstream applications [Dhaliwal, 2013]. Also, this method is quite time-consuming since the precipitation of protein or purified DNA both need a long incubation time.

Besides, in the commercial kits, silica-based method is mainly employed. DNA adheres specifically to silica membranes/ beads/ particles in a low salty solution at a defined pH while the residual contaminants could be removed by wash as a following treatment [Tan & Yiap, 2009]. Chaotropic agents are applied to denature protein and same as before, the precipitated sediments could be removed by centrifugation. This method is always combined with spin columns and microchips, which makes this procedure easier to operate than phenol/chloroform method. This method is cost-effective and suitable for automation, which is one reason for its application in most commercial kits [Tan & Yiap, 2009]. FastDNA Spin kit for Soil kit from MP Biomedicals is a example kit with this method.

Magnetic beads are another method for purification based on the binding between DNA and magnetic surface/ beads/ particles. These magnetic substances are coated with DNA-binding antibodies or functional groups that could specifically bind to DNA [Akbarzadeh et al., 2012]. Similar with silica-based method, after the DNA-binding, contaminants should be separated from beads and washed for removal. Finally, purified DNA is eluted with ethanol extraction [Ma et al., 2013]. This method is rapid and automated, but more expensive than other methods. One of the commercial kits that applied this method and would be evaluated in our research is Quick-DNA HMW MagBead kit (ZYMO Research, USA).

There still exist other purification methods, such as anion exchange [Budelier & Schorr, 1998] and salting out method [Jia et al., 2020]. The extraction method often needs to be improved and optimized for different kinds of DNA. Moreover, based on different lysis mechanisms or purification mechanisms used in each method, the quantity and quality of extracted DNA might be different. Past researches and their results on different evaluation methods are

discussed in the following sections.

1.3 Evaluation of DNA extraction methods

Hwang et al. [2009] did a similar research to evaluate the methods for the DNA extraction from drinking water distribution system biofilms. They compared two commercial kits, FastDNA Spin kit for soil and Powersoil DNA kit and three standard phenol/ chloroform methods from Schmidt, Zhou and Miller. Hwang et al. [2009] argued that the highest molecular mass DNA was obtained with protocols from FastDNA, Schmidt, and Zhou. Phenol-chloroform-based methods yield higher DNA concentrations than FastDNA Spin while the FastDNA Spin kit gives the best DNA purity. In all, FastDNA Spin kit for soil is recommended by Hwang for its reproducible results and fewer wastes produced during the process.

However, Hwang's research aimed at using DNA extracted for 454 pyrosequencing technique, which is the second generation sequencing technique. While the third-generation sequencing is focused more at present, to better suitable for it, there should be different preferences or requirements on the extracted DNA quality with the second-generation sequencing method.

Considering soil and sediment samples, Miller et al. [1999] evaluated and optimize the DNA extraction and purification procedures. She compared the beads mill and freeze-thaw technique as two physical treatments used for direct lysis and concluded that beads mill homogenization superior to freeze-thaw technique. However, in this research, only yield and purity of DNA was considered as evaluation criteria. Since beads mill might cause stronger shearing, a higher yield might relate with a lower high molecule weight of DNA. Besides, Miller et al. [1999] found that chloroform or phenol used in the lysis mixture would yield more DNA than those without organic solvents procedures.

Abd-Elsalam et al. [2011] modified a freeze-thaw based method for activated sludge which freezing the samples at -20C for 14h with lysis buffer [100 mM tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0) and 100 mM NaCl] and protease. This modified method applied both physical and chemical lysis methods and isopropanol for DNA precipitation. She compared this modified method with dBioZol kit (Bioer Technology Co., Ltd., China). The extracted DNA yield was found higher and fragment size longer than the selected commercial kit.

Furthermore, S. Sharma et al. [2019] compared the effects on extracted DNA of different reagents or temperature used in freeze-thaw techniques. Taken 0 °C, -20 °C, -80 °C as three freezing different temperatures, she concluded that with sterilize distilled water as buffer, freezing the samples at -20 °C overnight and thawing at 37 °C shows the highest yield while with phosphate buffered saline (PBS) as buffer, -80 °C freezing performs best in extracted DNA yield.

Solize Vosloo [2019] modified the Dneasy Powerwater kit and stated that the yield of extracted DNA improved at least two to three-fold. This method combines enzymatic, chemical and mechanical lysis mechanisms. The main difference between the modified method and original kit is the addition of lysozyme in the lysis buffer and the extra homogenization procedure, which means these two procedures might significantly improve the yield. However, compared with routine protocol, the time-consuming procedures for enzymatic and chemical lysis methods prolong the whole extraction time. Henne et al. [2012] also combined extra enzymatic and chemical lysis procedures with traditional commercial kits to increase the yield.

1.4 Research objectives

Our research is aimed to evaluate and modify these methods for DNA extraction for tap water samples to a better fit for subsequent sequencing analysis.
1. What's the difference of the quantity of the DNA extracted from drinking water samples using different DNA extraction methods?
2. What's the difference of the quality of the DNA extracted from drinking water samples using different DNA extraction methods?

2 Methodology

2.1 Experiment design

The whole experiments designed is shown as Fig 1. Tap water was filtered over the membranes firstly to collect the biomass inside. In all nine methods were selected out from past researches for evaluation and comparison. Four widely used commercial kits, three phenol/chloroform based extraction methods with different lysis procedures and one modified method according to the standard commercial kits, all these methods for DNA extraction were evaluated in this research with tap water samples.

Four commercial kits were selected to be tested in our research. FastDNA Spin kit for Soil (MP Biomedicals, USA) uses the mechanical lysis methods and purifies the extracted DNA with silica-based spin filter column, which is a rapid, efficient and highly reproducible method. The second and third kits are DNeasy PowerSoil kit (QIAGEN, USA) and DNeasy Powermax soil kit (QIAGEN, USA). These two methods both apply beads for vortex as mechanical lysis and inhibitor removal technology with spin

filter column. Also, DNeasy Powermax soil kit is suitable for DNA recovery from low biomass samples within large sample quantities, which corresponding to characteristics of tap water samples. The comparison between these two kit could compare the influence of samples quantities on the extracted DNA. Quick-DNA HMW MagBead kit (ZYMO Research, USA) is designed for high molecule weight DNA extraction, which applies enzymatic and chemical lysis as the replacement of beads beading to decrease the DNA shearing.

Different phenol/chloroform based direct lysis extraction methods were selected from literature, which used different procedures and reagents. Protocol of Zhou ([Zhou et al., 1996]), which is recommended by Hwang et al. [2009], was processed in our experiments. Modified ones with the addition of freeze-thaw lysis method were also approached and evaluated in our research. Based on Zhou's method, two kinds of freeze-thaw lysis methods reference to Abd-Elsalam et al. [2011] and S. Sharma et al. [2019] were selected as modifications. Besides, the modified method of Solize ([Solize Vosloo, 2019]) was also considered in this research.

To examine the reproducibility and reliability of the findings, each method was operated with triplicated samples. All methods used the same sample collected from the same tap. After DNA extracted, their yield, purity and fragment size were measured as the evaluation standards of different methods.

The quantification of the extracted DNA yield was assessed by the Qubit assay based on the principle of DNA-selective fluorescent dyes while the qualification was assessed by its purity and fragment size. The purity of DNA was evaluated by spectrophoto metric A260/A280 and A260/A230 ratios based on ultraviolet absorbance technology. A260/A280 ratio assesses the protein contamination of DNA which value greater than or equal to 1.8 shows pure DNA [Held, 2001]. A ratio of 260nm to 230nm could evaluate the level of salt carryover in the extracted DNA which value should be greater than 1.5, ideally close to 1.8 for pure DNA [Luebbehusen, 2006]. Besides, the A260/A230 ratio values might be higher than the respective A260/A280 ratio values for better purity.

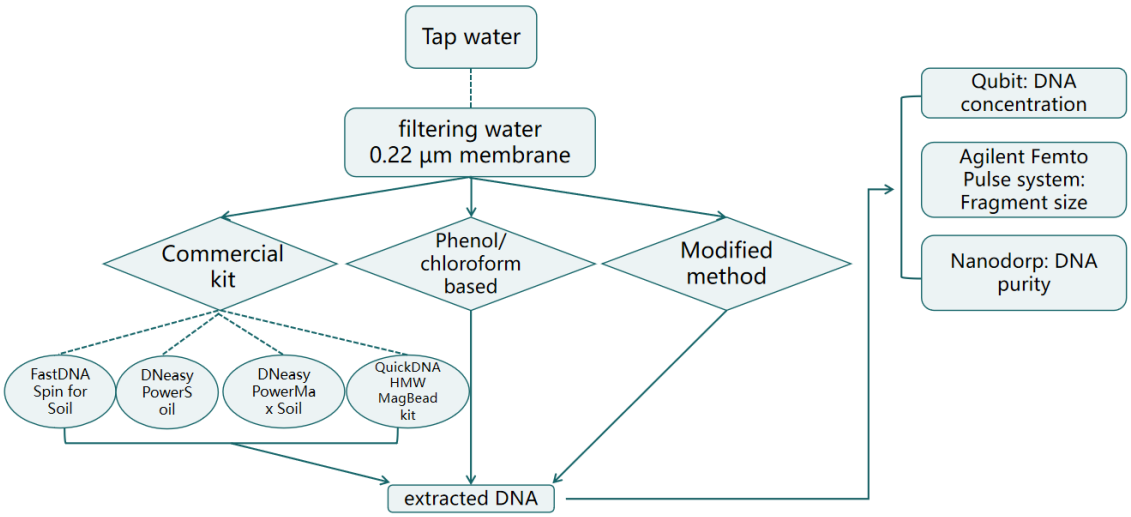

Figure 1: FLow chart

2.2 Extraction methods

To ensure the reproducibility of samples, all tap water samples were collected from the same tap, which locates at WaterLab in Faculty of Civil Engineering and Geosciences, TU Delft. Before sampling, this tap was kept running for over 2 minutes to eliminate the influence of disturbance and loose deposits in the distribution system on the water quality. Vaccum filtration was applied to concentrated microbes by filtration through 0.22 μm pore size Polyether sulfone (PES) filters (Millipore Express® PLUS, USA). According to the biomass measured in fluid tap water, the amount of filtered tap water was set as 50 to 70 liters per membrane. Different methods conducted with various number of membranes according to the biomass suggested in their standard protocols. The collected filter membranes were stored in a petri dish at -20°C. For mostly methods, filter membranes was cut into small pieces by sterilized scissors as a pre-treatment to increase the contact with lysing matrix of various methods for higher efficiency. Table 1 shows the different lysis and purification methods of various methods and the following paragraphs discuss the specific protocol of each method approached in this research.

FastDNA Spin Kit for Soil (MP Biomedicals, USA)
Modifications were done with standard protocols to improve the quality and quantity of extracted DN. After homogenization with MiniBeadBeater-16 (BioSpec, USA) for 40 s, incubate the samples at room temperature for 30 min could reduce the DNA shearing. Then, centrifugation was prolonged to 15 min for better removal of debris. To eliminate the protein contamination, 5 mins' incubation on ice with PPS solution is necessary. Wash steps with SEWS-M solution was repeated as three times and followed air dry of the Spin Filter was enhanced with 60 °C incubation for higher purity. Finally, to eluate DNA, with DES covered, the binding matrix was performed at 55°C for 5 min.

DNeasy PowerSoil (QIAGEN, USA)
According to the standard protocol and troubling shooting provided by QIAGEN, to decrease DNA shearing, the vortex mixing for 10 min was replaced by vortex for 30s and incubation at 70°C for 5 min. And this procedure was repeated for three times. Also, the filter column was air dried for 5 min at room temperature for better purity and after the final solution was added, incubation at 55° for 5 min before centrifugation for a higher yield. Besides, the final solution added was decreased to $50\mu l$ to increase the concentration.

DNeasy PowerMax Soil (QIAGEN, USA)
Extraction procedures were almost same with the standard protocol and the vortex mixing used Vortex-Genie 2 (Thermo fisher scientific, USA) with the maximum speed as 3200 rpm. Also, after wash steps, air dry for 5 min was added for the removal of organic contents. Since the final volume of extracted DNA was quite large but concentration was low, the samples were decided to be concentrated with isopropanol. With 0.6 volume of isopropanol, the solution was incubated at room temperature for 1 h for DNA precipitation. 70% cold enthanol was used for precipitated DNA wash. Then, the samples were air dried for 5 min at room temperature before final dissolving.

Quick-DNA HMW MagBead kit (ZYMO Research, USA)
Since this kit was a quite new one, a pre-experiment was done with it followed the standard protocol for soil samples. With the measurement of the pre-experiment result (not shown in this article), some modifications to increase the yield and purity was done. Briefly, to fully merge the cut membrane, the lysis buffer and following solutions were all doubled their volume. The incubation time for protein digestion was extended from 10 min to 2 h for the higher purity. With the MagBing Beads, the samples were mixed by Vortex-Genie 2 (Thermo fisher scientific, USA) with the vortex speed setting as level 3 around 1400 rpm for 10 min and incubated at room temperature for 10 min. The times of the following mixing steps by pipette were doubled. To dry the beads, the samples were heated at 55 °C for 10 min. The samples were also incubated at 55 °C for 5 min with elution buffer to increase the yield.

Phenol/chloroform method with chemical lysis
Zhou approached chemical lysis with SDS-based DNA extraction method. Considering the biomass of collected tap water samples, the specific dose of each reagents were modified. Each membrane was consistent with $1.35ml$ DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB) and $10\mu l$ proteinase K (10 mg/ml), vortex for mixing and 30 mins at 37°C incubation followed. Then $150\mu l$ of 20% SDS was added, and the samples were incubated in a 65°C for 2 h with gentle inversions every 15 to 20 min. Centrifugation at 10000 rpm at room temperature for 10 min to separate debris of cell lysis. Supernatants were saved in a new tube and pellets were extracted two more times with $450\mu l$ of the extraction buffer and $50\mu l$ of 20% SDS. Each time vortex mix for 10 s, incubate at 65°C for 10 min, and centrifuge as before. Supernatants from the three cycles of extractions were combined for the following purification procedure with chloroform/isoamyl alcohol (24:1,vol/vol). 0.6 volume of isopropanol was applied for the aquatic phase for DNA precipitation and 70% cold ethanol was used for pellets wash.

Phenol/chloroform method with physical lysis
Same DNA extraction buffer as chemical lysis and freeze-thaw technique was chosen as the physical lysis method. After lysis buffer was added and mixed with cut membranes, two kinds of freeze-thaw procedures were applied. One procedure was that the samples were incubated at -20°C overnight and thaw at 37°C for 30 min. While the other one used cycled freeze-thaw technique, with samples frozen at -20°C for 30 min and thawed at 60°C for 10 min as one cycle, repeated for three times. Then $150\mu l$ of 20% SDS was added, and the samples were incubated in a 65°C for 2 h with gentle inversions every 15 to 20 min. Centrifuge and keep the supernatants for the following steps, removal of protein and precipitation of DNA.

Modified method
According to Ameet's method, Powerwater kit was combined with enzymatic lysis method. For better comparison of this method with standard commercial kits, Powersoil kit took replacement of Powerwater kit in our research. There also existed other modifications. With cut membrane inside the Lysing

matrix E tube without beads, 500μl of DNA extraction buffer was add (10× Tris-EDTA buffer[100 mM Tris, 10mM EDTA], 10mg/ml lysozyme solution), mixed by vortex and incubated at 37°C for 30 min. After this treatment, 300μl C1 (DNeasy PowerSoil kit) and 85μl proteinase K (10mg/ml) was added and the samples were incubated at 65°C for 30 min. Aseptically transfer the beads back to the corresponding Lysing Matrix E tubes. The supernatants was pipette off, kept in a new tube and mixed with an equal volume of chloroformisoamyl alcohol (24:1, vol/vol). The mixture was transferred back to Lysing Matrix E tubes and homogenized with MiniBeadBeater-16 (BioSpec, USA) for 40 s and centrifuge at 14000 $\times g$ for 10 min. The supernatants was kept for following procedures, which followed the protocol of DNeasy PowerSoil kit from step 9. After wash step with C5, an extra wash with 70% ethanol was added. Similar with modifications of this protocol above, air dry and incubation were added and final solution amount was decreased to 50μl.

Table 1: Details of selected DNA extraction methods

Method	Code	Lysis mechanism	Purification mechanism
Dneasy powersoil kit	C1	Mechanical (Beads beating with vortex) and chemical lysis	Inhibitor removal technology with spin filter columns
Dneasy powermax soil kit	C2	Mechanical (Beads beating with vortex) and chemical lysis	Inhibitor removal technology with spin filter columns
FastDNA Spin kit for Soil	C3	Mechanical (Beads homogenization) and chemical lysis	Silica-based method with spin filter columns
Quick-DNA HMW MagBead kit	C4	Enzymatic lysis and chemical lysis(lysozyme incubation at 37°C for 30 min, followed by SDS, proteinase K, high temperature incubation at 55°C for 2h	Magnetic beads method
Modified method from Solize Vosloo [2019]	M	Enzymatic lysis and chemical lysis(lysozyme incubation at 37°C for 60 min, followed by SDS, proteinase K, high temperature incubation at 55°C for 30 min and Mechanical (Bead homogenization)	Inhibitor removal technology with spin filter columns
Zhou et al. [1996]	Z1	Chemical lysis (proteinase K incubation at 37°C for 30 min, followed by SDS, high salt, and high temperature incubation, 65°C for 2 h)	Phenol/chloroform method
Freeze-thaw based on Zhou et al. [1996]	Z2	Physical lysis (freeze at -20°C overnight and thaw at 37°C for 30 min) and chemical lysis	Phenol/chloroform method
Freeze-thaw cycle based on Zhou et al. [1996]	Z3	Physical lysis (freeze at -20°C and thaw at 60°C for three cycles) and chemical lysis	Phenol/chloroform method

2.3 Measurement and assessment of extracted DNA and methods

Quality and purifiation of extracted DNA was sent to Leiden University Medical Center for measurement. The Agilent Femto Pulse System was applied for fragment size measurement as the criteria of their quality. DNA purity was assessed with use of A Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) via absorption ratios of the extracts at A260/A280 and A260/A230. Also, the quantity of extracted DNA could be measured at the same time. However, spectrophotometer measurements could be influenced by contaminants (e.g. free nucleotides, salts, and organic compounds) and not sensitive to low DNA concentrations. Thus, DNA concentration of extracted DNA samples was quantified with Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA) as a complement assessment. Following the manufacturer's instructions, dsDNA High Sensitivity Assay kit (Thermo Fisher

Scientific, USA) was selected as the suitable kit for the samples. Reproducibility was evaluated by the deviation of triplicated samples of each method. Operational convenience of various methods was graded according to the time-consuming (mainly caused by incubation time) and reagents availability, such as the preparation of the needed reagents or the dangerous degree of the used reagents.

3 Results & Discussions

According to the evaluation criteria, extracted DNA yield, purity, fragment size would be discusses as below and the reproducibility operational convenience of each method might also be concluded considering different aspects.Table 2 shows the yield and purity of the extracted DNA with different methods.

Table 2: Yield and purity of extracted DNA

Samples	Methods Code	Yield [ng]	Purity	
			A260/A280	A260/A230
membrane filtered 70L	C1	9.63 $ng/\mu l$ * 50 μl	1.6	0.7
membrane filtered 70L	C1	10.2 $ng/\mu l$ * 50 μl	1.7	0.6
membrane filtered 70L	C1	10.9 $ng/\mu l$ * 50 μl	2.1	1.1
membrane filtered 50L*4	C2(no concentrated)	1.99 $ng/\mu l$ * 1000 μl	-	-
membrane filtered 50L*4	C2(no concentrated)	2.02 $ng/\mu l$ * 1000 μl	-	-
membrane filtered 50L*4	C2(no concentrated)	1.84 $ng/\mu l$ * 1000 μl	-	-
membrane filtered 50L*4	C2-1	8.94 $ng/\mu l$ * 50 μl	1.9	1.6
membrane filtered 50L*4	C2-1	3.03 $ng/\mu l$ * 50 μl	-	-
membrane filtered 50L*4	C2-1	9.39 $ng/\mu l$ * 50 μl	1.9	1.5
membrane filtered 70L	C3	23.7 $ng/\mu l$ * 50 μl	1.6	0.3
membrane filtered 70L	C3	20.5 $ng/\mu l$ * 50 μl	1.6	0.4
membrane filtered 70L	C3	20.4 $ng/\mu l$ * 50 μl	1.5	0.7
membrane filtered 70L*3	C4-2	7.72 $ng/\mu l$ * 50 μl	1.2	0.6
membrane filtered 70L*3	C4-2	7.85 $ng/\mu l$ * 50 μl	0.8	0.5
membrane filtered 70L*3	C4-2	9.39 $ng/\mu l$ * 50 μl	0.9	0.4
membrane filtered 70L	C4-3	51.0 $ng/\mu l$ * 60 μl	1.6	1.3
membrane filtered 70L	C4-3	50.0 $ng/\mu l$ * 60 μl	1.6	1.2
membrane filtered 70L	C4-3	53.0 $ng/\mu l$ * 60 μl	1.6	1.2
membrane filtered 20L	M	16.5 $ng/\mu l$ * 50 μl	1.2	0.9
membrane filtered 20L	M	17.9 $ng/\mu l$ * 50 μl	1.2	0.9
membrane filtered 20L	M	21.5 $ng/\mu l$ * 50 μl	1.1	1.1
membrane filtered 70L	Z1	14.6 $ng/\mu l$ * 50 μl	1.8	0.7
membrane filtered 70L	Z1	23.5 $ng/\mu l$ * 50 μl	2.1	1.6
membrane filtered 70L	Z1	19.1 $ng/\mu l$ * 50 μl	2.1	1.5
membrane filtered 70L	Z2	23.9 $ng/\mu l$ * 50 μl	2.1	2.1
membrane filtered 70L	Z2	31.7 $ng/\mu l$ * 50 μl	2.1	2.1
membrane filtered 70L	Z2	26.9 $ng/\mu l$ * 50 μl	2.1	2.2
membrane filtered 55L	Z3	28.9 $ng/\mu l$ * 50 μl	2.1	2.1
membrane filtered 55L	Z3	20.4 $ng/\mu l$ * 50 μl	2.1	2.2
membrane filtered 60L	Z3	39.4 $ng/\mu l$ * 50 μl	2.1	2.1

3.1 Quantity of extracted DNA

Extraction efficiency of different methods are shown in the Fig 2 as below. Different methods were consistent with different sample and final solution systems, as a result, for better comparison, the extraction efficiency of each method was calculated. Extraction efficiency is the extracted DNA amount of within per liter of water filtered through the membrane with the unit as ng/L.

Table 3: Average extraction efficiency & RSD of different methods

Methods Code	Extraction efficiency [ng/L]	RSD [%]
C1	7.32 ± 0.45	6.21
C2	10.36 ± 0.59	5.66
C2-1	1.97 ± 0.99	50.32
C3	15.38 ± 1.34	8.72
C4-2	1.98 ± 0.22	11.16
C4-3	44.00 ± 1.31	2.98
M	46.58 ± 6.45	13.84
Z1	13.62 ± 3.18	23.34
Z2	19.64 ± 2.81	14.31
Z3	25.88 ± 7.15	27.63

It is shown below that the Modified method (M) gave the highest extraction efficiency as the average efficiency around 47 ng/L and the highest one sample even over 50 ng/L, while the third version of Quick-DNA HMW MagBead kit (C4-3) followed as 44 ng/L as the average value. Then, two phenol/chloroform based methods with different freeze-thaw techniques appears as the third and fourth highest extraction efficiency which specifically are 19.64 ng/L and 25.88 ng/L. The other two methods (C3, C2) show a similar extraction efficiency around 15 ng/L. Before concentrated, samples extracted with Dneasy Powermax soil kit (C2) show larger extraction efficiency than Dneasy Powersoil kit (C1) (10.36 ng/L $>$ 7.32 ng/L), and the significant difference ($p = 0.03928 < 0.05$) between these two kits states that the increased input quantities could increase the final extracted DNA yield for tap water samples. The second version of Quick-DNA HMW MagBead kit (C4-2) which applied ultrasonic separation method owns the lowest efficiency even less than 2 ng/L. Between these two versions of Quick-DNA HMW MagBead kit, the extraction efficiency has significant difference within 95% confidence interval ($p = 0.00095 < 0.05$), which convinced that the separation method with ultrasonic has a negative effect on the extraction efficiency of tap water samples.

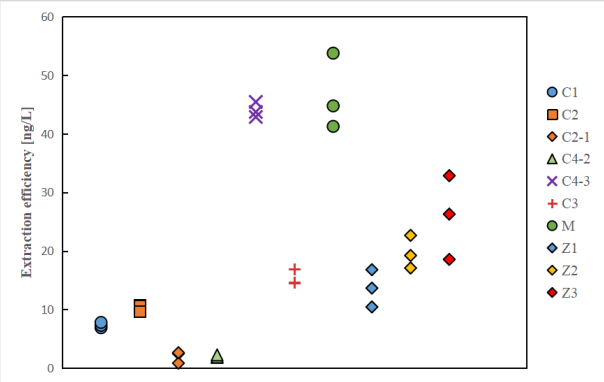

Figure 2: Extraction efficiency of samples from different methods

What is more, the error bar in Fig 3 shows the reproducibility on the extraction efficiency of each method. For better comparison among all these methods, not only standard deviation of triplicated samples, but also the relative standard deviation (RSD) were calculated and shown in the Table3. Concentration step in Dneasy Powermax soil kit (C2) not only causes significant loss in extraction efficiency ($p = 0.014 < 0.05$), but also lowers the reproducibility of this method. Despite that the second version of Quick-DNA HMW MagBead kit shows a quite low standard deviation, the RSD is larger. Instead, the third version of Quick-DNA HMW MagBead kit gave the least RSD even less than 3%, which means that this method has the best reproducibility on extraction efficiency. Then, the Dneasy Powermax for soil and Dneasy Powersoil kit show the second and third least RSD, respectively as 5.66% and 6.21%, corresponding to the second and third best reproducibility. Another interesting finding here is that the commercial kits methods all show the less RSD than phenol/chloroform based methods (including Z1, Z2, Z3), which is consistent with a better reproducibility than others.

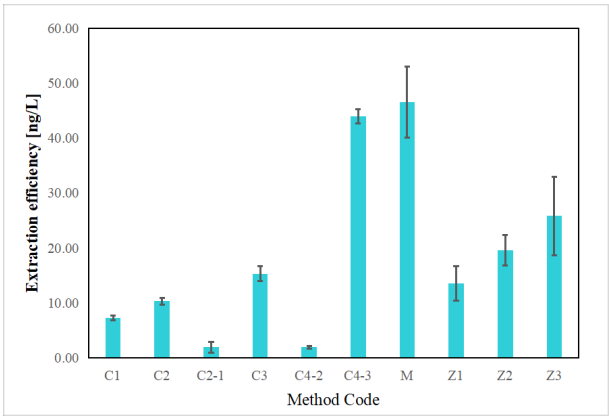

Figure 3: RSD of different methods on Extraction efficiency

In the aspect of various lysis mechanisms of different methods, the comparison among different methods could be found in Table 1. It is obvious that enzymatic lysis give a higher efficiency than chemical and physical ones, while the one with mechanical lysis methods shows the lowest efficiency. Considering the comparison among three phenol/chloroform based methods, the addition of freeze-thaw techniques greatly improves the extraction efficiency, especially the one used cycled freeze-thaw techniques. Freeze-thaw techniques give a positive effect on the yield of extracted DNA.

The two methods which performed enzymatic lysis mechanisms show higher extraction efficiency, and the modified methods with followed bead homogenization as mechanical lysis is the highest one. Beads homogenization might help improve the efficiency, but here this mechanical lysis methods does not give a significant difference between these two methods ($p > 0.05$). One possible reason which results for this phenomenon is the different buffer in this two methods. Consequently, a higher working concentration of lysozyme in Quick-DNA HMW MagBead kit might cause a higher lysing efficiency, which decrease the gap caused by the extra mechanical lysis procedure. Besides, there exists significant difference within 95% confidence interval ($p = 0.01147 < 0.5$) in the extraction efficiency between modified method (M) with Dneasy Powersoil kit (C1), which might caused by the addition of enzymatic and chemical lysis in the modified method.

A problem risen during the yield comparison among various methods is related to the used evaluation criteria. The prerequisite for extraction efficiency calculation is that the amount of biomass intercepted on the membrane should be a linear correlation with the amount of water filtered. However, this assumption has not been convinced. It is possible that the intercepted biomass amount on the membrane could be a curve correlation with filtered water amount.

3.2 Quality of extracted DNA

The quality of extracted DNA are evaluated with their purity and fragment size.

3.2.1 Extracted DNA purity comparison

The DNA purity was measured as A260/A280 and A260/A230 ratios for protein or salt contaminants. The averaged measured ratios of different methods are shown in Table 4 and corresponding graphs in Fig 4.

Table 4: Average purity of different methods

Methods Code	A260/A280	A260/A230
C1	1.80 ± 0.26	0.80 ± 0.26
C2-1	1.75 ± 0.21	1.70 ± 0.28
C3	1.57 ± 0.06	0.47 ± 0.21
C4-2	0.93 ± 0.15	0.50 ± 0.10
C4-3	1.60 ± 0.00	1.23 ± 0.06
M	1.17 ± 0.06	0.97 ± 0.12
Z1	2.00 ± 0.17	1.27 ± 0.49
Z2	2.10 ± 0.00	2.13 ± 0.06
Z3	2.10 ± 0.00	2.13 ± 0.06

For all methods, the A260/A280 ratio values are more closer to the ideal value than A260/A230 ratio, which means protein contaminants are more easily to be digested and removed. As shown in the Fig 4, DNA extracted with method from Zhou et al. [1996] give the best purity for both two ratio index, especially for A260/A280 ratio which even have an ideal value above 1.8. It could be argued that phenol/chloroform based purification methods thoroughly remove the protein and organic contaminants from DNA. Comprehensively considering the two purity index, Dneasy Powersoil kit shows the second highest purity. Extracted DNA from FastDNA Spin kit for soil have similar values for A260/A280 ratio with Dneasy Powersoil kit ones while the averaged A260/A230 ratio is lower. For the two versions of Quick-DNA HMW MagBead kit, the purity of third version is significantly higher than the second version ($p_{A260/A280} = 0.0044 < 0.05$, $p_{A260/A230} = 0.0044 < 0.05$), which means that the prolonged incubation time for protein digestion and magnetic beads adsorption could help improve the purity. Comparison between modified method and Dneasy Powersoil kit, despite the modified procedures improves the extraction efficiency, the purity decreases, especially for protein contaminants. This phenomena might cause by the addition of organic solvents and limited incubation time for protein digestion. The added concentration step in Dneasy Powermax soil kit, compared with Dneasy Powersoil kit, is beneficial for the purity, which shows a higher value in A260/A230 and a similar value for A260/A280.

What is more, compared among all methods with various purification mechanisms, the phenol/chloroform method performs best in purification. Then, the methods which apply spin-filter columns combined with different binding matrix shows the higher efficiency in purification than magnetic beads method. Despite both methods use the spin-filter columns, samples extracted from two Dneasy kits (QIAGEN, USA) have better purity than samples extracted with FastDNA Spin kit for Soil.

The reproducibility of extracted DNA purity is varied. Mostly, the deviations of A260/A280 are smaller than deviations of A260/A230 for each method, which describes that The reproducibility in protein removal is better. Thus, both the average value and reproducibility of protein removal are more effective and easier to be operated than salt residuals. Among all methods, these two freeze-thaw based phenol/chloroform methods show both the best purity and reproducibility of purity. Beside, the third version of Quick-DNA HMW MagBead kit shows a high reproducibility of purity and relatively medium purity.

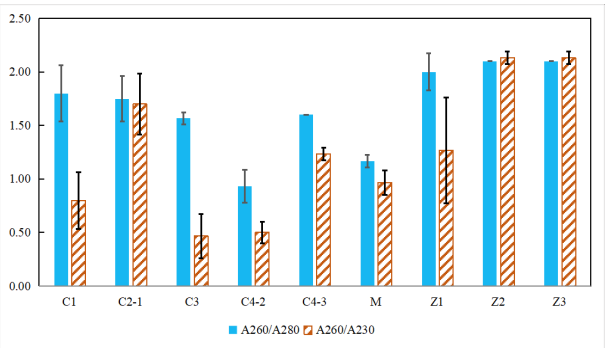

Figure 4: Purity of samples from different methods

3.2.2 Extracted DNA fragment size comparison

Two indexes are considered here for fragment size, one is the peak size, which accounts for the tallest/most concentrated portion of the sample. The other is the smear size, which is the distribution of the concentration over the designated smear range. This measurement was done by Leiden University Medical Center by the Agilent Femto Pulse system. This system is designed for high molecular weight (HMW) gDNA quality and size measure-

ment. Compared with traditional pulsed-field gel electrophoresis analysis, it is less time-consuming and extreme sensitive.

Table 5: Fragment size of samples with different methods

Method code	Peak [bp]	Smear Range [bp] Low	High
C1	3326	1070	21566
	3226	69	24001
	3801	879	18569
C2-1	6625	1065	19900
	-	-	-
	6200	1070	17392
C3	7525	937	23601
	7400	971	23334
	7447	920	22600
C4-2	-	-	-
	2576	244	168756
	3126	236	73972
C4-3	13774	936	130003
	9504	934	167083
	10970	987	178698
M	11021	1107	29017
	11582	1129	35287
	12703	1102	37124
Z1	796	1222	22960
	10409	3432	19602
	12805	2590	32690
Z2	4225	767	25028
	10113	1167	47657
	9010	1175	33587
Z3	11396	1136	32395
	10224	2499	44183
	9818	1187	20938

The peak and smear size of each sample are summarized in the Table 5. The averaged peak size and relatively standard deviation for triplicated samples for each method is shown in Fig 5. The peak size is the corresponding fragment size with the highest peak in each electrophergram. It is obvious that the modified method (M) and the third-version of Quick-DNA HMW MagBead kit (C4-3) have the relatively longer fragment size as the peak size, which means the extracted DNA from these two methods more concentrated in larger size and better quality. Besides, the third-version of Quick-DNA HMW MagBead kit (C4-3) performs much better than the second version (C4-2) in extracted DNA quality, with significantly larger peak size in samples. Compared with Dneasy Powersoil kit (C1), the modified method (M) shows a significant improvement in the peak size of extracted DNA ($p = 0.0053 < 0.5$), which means the organic solvents added in the mechanical lysis might help decrease the shearing of DNA. What is more, the methods with chemical and enzymatic lysis mechanisms mainly result in a larger peak size of the samples fragment. These kinds of lysis mechanisms could significantly lower DNA shearing and lead to more concentrated long fragment. These three phenol/chloroform based methods show the similar averaged values of the peak size with the highest two methods (M & C4-3), but much less reproducibility in this aspect.

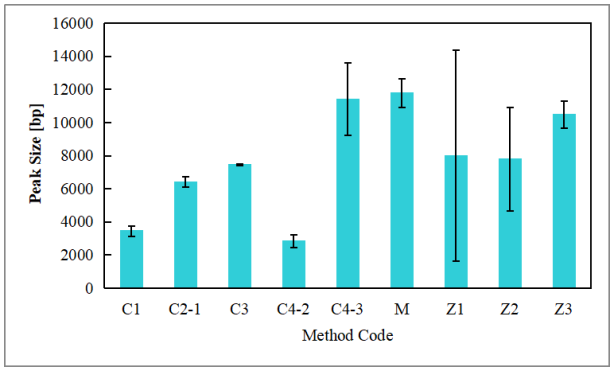

Figure 5: Peak size of samples from different methods

Since the reproducibility in smear size of triplicated samples is acceptable, gel-image of one sample for each method was selected out and shown in Fig 6 for better comparison. The smear ranges of samples are summarized from eletrophergrams. To avoid the effect of complex and small zones, only when corresponding corrected peak areas were larger than 10, the zones were considered as main smear range. Based on this standard, the smear range of each samples were concluded in Table 5.
The three traditional commercial kits, along with the modified method (M) based on the commercial kits, show the similar smear ranges. The modified method (M), which shows a concentrated fragment size distribution with high peak sizes, should be the best performance in DNA quality and size among these four methods. The two versions of Quick-DNA HMW MagBead kit have a much wider smear size than other methods, even longer than 100 kb, which confirms its ability to extracted HMW DNA. With comparison between these two versions from Fig 6, the third-version has less concentrated short fragments, which is more recommended for HMW DNA extraction. Unlike the bell-shaped smears in the common methods with only one peak, the

phenol/chloroform methods (Z1, Z2, Z3) have two peaks, one is in the quite short fragment zones, even less than 1 kb. Consequently, the further size selection procedures are important for these methods to remove the short fragments. Among these three methods with various lysis mechanisms, additional freeze-thaw techniques widens the smear range. However, more short fragments exist followed with the wider smear sizes.

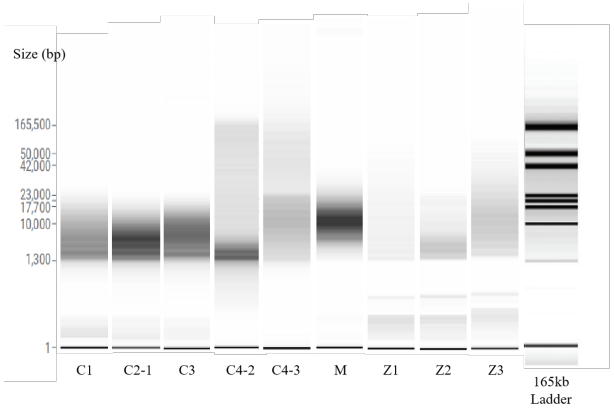

Figure 6: Gel-image from the Agilent Femto Pulse system of samples from different methods

3.3 Operational convenience of methods

Operational convenience also should be considered as one criteria to evaluate various methods. One of the main point is the possible consumed time of different methods, especially the methods with chemical or enzymatic lysis procedures, always along with long incubation time. Here, the necessary incubation time are used as the reference for the whole consumed time of each method.

Table 6: Operational convenience comparison of different methods

Method code	Necessary incubation time (approximaty)	Extra reagents	Hazardous waste
C1	30 min		
C2	50 min		
C2-1	150 min	√	
C3	70 min		
C4-2	180 min	√	
C4-3	180 min	√	
M	120 min	√	√
Z1	260 min	√	√
Z2	270 min (ignore overnight time)	√	√
Z3	360 min	√	√

The three phenol/chloroform based methods are most time-consuming, especially for the freeze-thaw cycle one. Then, the Quick-DNA HMW MagBead kit (C4) and Modified method (M) show the second two longest operational time, since the chemical and enzymatic lysis mechanisms they applied needs enough incubation time than mechanical lysis mechanisms based methods. Also, the purification and followed DNA precipitation procedures in the phenol/chloroform based methods both needs long centrifugal time for thorough separation. Comparing between the Quick-DNA HMW MagBead kit (C4) and Modified method (M), the modified one was less time-consuming with short incubation time with proteinase K.

Besides the time-consuming, the reagents preparation and possible wastes are the other point for operational convenience. It is obvious that the commercial kits should be more convenient in reagents preparation than modified (M) and phenol/chloroform based ones (Z1, Z2, Z3). However, considering the water sample we used, the Quick-DNA HMW MagBead kit (C4) also needs some extra preparation processes and corresponding reagents besides the standard kits. The self-prepared reagents or solvents also lowered the reproducibility of these methods, because such procedures highly dependent on the experimenters' operational stability. The other problem is caused by the possible waste. The modified methods and phenol/chloroform based methods

applied organic solvents, which led to possible hazardous wastes and increased the operational risk.

Consequently, Dneasy kits (C1, C2) and FastDNA Spin Kit for Soil (C3) shows the highest operational convenience, followed by Quick-DNA HMW MagBead kit (C4), which needs extra reagents and some incubation time. Then, although all needs extra reagents preparation and related hazardous wastes, the modified method (M) gave a better performance than phenol/chloroform ones, because of less time-consuming and relatively less extra reagents besides standard kits.

4 Conclusion

According to the results presented above, the various DNA extraction methods of tap water samples have various advantages and drawbacks. It could be concluded that the commercial kits all shows better reproducibility and easier operated protocols than modified or phenol-chloroform based methods. The third-version of Quick-DNA HMW MagBead kit (C4-3) performs best in extracted DNA yield with high extraction efficiency and reproducibility. However, the unproved assumption under the yield comparison make this conclusion less convincing. While in the aspect of purity, the phenol/chloroform based methods, especially with freeze-thaw technique ones (Z2 & Z3), show the ideal purity and highest reproducibility. Furthermore, the modified method (M), whose fragment distributes concentrated with the highest peak size, show the better quality of DNA. While the third-version of Quick-DNA HMW MagBead kit (C4-3), which have a much wider distribution of fragment with more HMW DNA but less concentrated, also perform well in the quality of DNA. In the aspect of operational convenience for each method, the commercial kits would be more suitable than modified and phenol/chloroform based methods.

Comprehensively considering the yield, purity, quality of extracted DNA from each method and its reproducibility, the third-version of Quick-DNA HMW MagBead kit (C4-3) is suggested, despite the purity of extracted DNA is not ideal, but the yield and quality is both performs the best, especially for the large amount of extracted HMW DNA (>50 kb). The existence of HWM DNA is beneficial for the following sequencing. The other drawback of this method is caused by its operation. Since the long incubation time during the protocol, this method is more time-consuming than other commercial kits. The modified method (M) should be recommended for a bit less time-consuming. This method shows a similar high yield and a concentrated fragment distribution with high peak sizes. However, both these two methods need extra reagents besides the standard commercial kits. Thus, FastDNA Spin kit for Soil (C3) could be applied as the replacement for its convenient operation and rapid procedures. The phenol/chloroform based methods are not worthy for its complex operation, long incubation time and possible generated hazardous wastes, even they could give the ideal and best purity and relatively high yield. Dneasy Powersoil kit (C1) and Dneasy Powermax for soil kit (C2) both performed not very well in the evaluation, with low yield and low quality in fragment size.

However, the aim of our research is to find the optimal DNA extraction method for further study on tap water microbiology. As a result, the possible difference in the microbial communities caused by various methods should also be an important criteria, which is both the limit and further research prospect for our study. It is recommended that the optimal DNA extraction method needs to be carefully selected according to the specific requirements and the sample characteristics of the research.

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