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The guanidine thiocyanate-high EDTA method for total microbial RNA extraction from severely heavy metal-contaminated soils

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Summary

Molecular analyses relying on RNA, as a direct way to unravel active microbes and their functional genes, have received increasing attention from environmental researchers recently. However, extracting sufficient and high-quality total microbial RNA from seriously heavy metal-contaminated soils is still a challenge. In this study, the guanidine thiocyanate-high EDTA (GTHE) method was established and optimized for recovering high quantity and quality of RNA from long-term heavy metal-contaminated soils. Due to the low microbial biomass in the soils, we combined multiple strong denaturants and intense mechanical lysis to break cells for increasing RNA yields. To minimize RNAase and heavy metals interference on RNA integrity, the concentrations of guanidine thiocyanate and EDTA were increased from 0.5 to 0.625 ml g^{-1} soil and 10 to 100 mM, respectively. This optimized GTHE method was applied to seven severely contaminated soils, and the RNA recovery efficiencies were 2.80 ~ 59.41 μg g^{-1} soil. The total microbial RNA of non-Cr(VI) (NT) and Cr(VI)-treated (CT) samples was utilized for molecular analyses. The result of qRT-PCR demonstrated that the expressions of two tested genes, *chrA* and *yieF*, were respectively upregulated 4.12- and 62.43-fold after Cr(VI) treatment. The total microbial RNA extracted from NT and CT samples, respectively, reached to 26.70 μg and 30.75 μg, which were much higher than the required amount (5 μg) for metatranscriptomic library construction. Besides, ratios of mRNA read were more than 86%, which indicated the high-quality libraries constructed for metatranscriptomic analysis. In summary, the GTHE method is useful to study microbes of contaminated habitats.

Introduction

Soil microbiota are a core of biogeochemical cycles, including nutrient cycling, mineralization and decomposition pathways, and the removal of pollutants (Gunnigle *et al*., 2014; Thorn *et al*., 2018). The appearance of meta-sequencing techniques has revolutionized research of soil microorganisms (Griffiths *et al*., 2000). DNA-dependent metagenomic analyses have been widely used to understand the genetic potential of microbial communities in environments (Ufarte *et al*., 2015). However, it cannot identify the active microbes and their genes (Couradeau *et al*., 2019). RNA responds promptly to the changing environmental factors (Hua *et al*., 2015). Thus, metatranscriptomic sequencing is a reliable way to reveal active microbial community members and their metabolic pathways. For instance, metatranscriptomic analysis of coniferous forest soil compared the active

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microbiota in topsoil and elucidated the producers of essential cellulose decomposition genes (*cbhI*), during organic matter decomposition (Baldrian *et al*., 2012). Another study revealed the effects of atmospheric CO_2 change on the rhizosphere microbiomes by metatranscriptomic sequencing in European grassland (Bei *et al*., 2019). Therefore, it is a promising way to study gene expression profiles of microbes in different soil habitats relying on microbial RNA.

The pressure of heavy metal-contaminated soil enriches highly efficient remediation microbes and genes, conducive to soil heavy metal contamination restoration (Epelde *et al*., 2015). However, studies based on the total microbial RNA to reveal the active microbes and their genes have been rarely reported in heavy metal-contaminated soils. It might be because of the methodological issue. In the past decade, many researchers explored soil microbes by metagenomic approach, while only a few of them investigated soil microbes by metatranscriptomic method, especially heavy metal-contaminated soil samples (Fig. S1). Heavy metals always coexist with organic pollutants, giving a low microbial biomass in soil under the severely contaminated conditions (Yang *et al*., 2006; Huang *et al*., 2016). In the metabolic processes, heavy metals and organic pollutants may cause harm to microbes by reactive oxygen species (ROS)-induced RNA damage, such as 7,8-dihydro-8-oxoguanine, heavy metal-RNA adducts, apurinic/apyrimidinic (AP) sites, and strand breaks (Salnikow and Zhitkovich, 2008; Santos-Escobar *et al*., 2014; Obermeier *et al*., 2015). Besides, heavy metal residues in RNA also interfere with the enzymes involved in the molecular operations, including PCR, microarrays, and metatranscriptomic sequencing. Also, RNA extraction from soil is harder than DNA due to the following reasons: (i) hydroxyl group of ribose and more free extracyclic functional groups in RNA than in DNA could form hydrogen bonds with soil surface, which result in stronger attachment of RNA on soil particles (Robinson *et al*., 2007; Cleaves *et al*., 2010); (ii) the unstable single-stranded structure of RNA; (iii) the ubiquitous and unextinguished RNAase (Dineen *et al*., 2010).

Currently, RNA applications such as qRT-PCR, microarrays, and metatranscriptomic sequencing require high amount and quality of RNA. Therefore, several methods have been developed for RNA recovery from soil including conventional methods and commercial kits. Although conventional methods are time-consuming, complicated, and have low RNA recovery efficiency, they can be modified to fit soil physiochemical characteristics (Lehembre *et al*., 2013). Commercial kits, such as Soil Quick RNA isolation Kit (Huayueyang, Beijing, China), Power Soil® Total RNA Isolation Kit (Mobio, Carlsbad, CA, USA), and Soil RNA Kit (Omega, Norcross, GA, USA), are used for total microbial RNA extraction from soil because of their quick process and positive result for molecular analyses compared with conventional methods (Wang *et al*., 2009). However, very few researches have reported the successful extraction of the total microbial RNA from heavy metal-contaminated soils by conventional methods or commercial kits (Lehembre *et al*., 2013; Epelde *et al*., 2015; Cabral *et al*., 2016). None of them could extract enough and high-quality total microbial RNA from NT and CT. Therefore, it is crucial to develop a highly efficient and robust RNA extraction method for exploring the active remediation microbes and their heavy metal remediation genes in seriously heavy metal-contaminated soils.

There are many influencing factors on RNA extraction efficiency in seriously heavy metal-contaminated soils, such as heavy metals, organic pollutants, microbial biomass, soil texture, humic acid, and RNase (Wang *et al*., 2009; Santos-Escobar *et al*., 2014; Thorn *et al*., 2018). In this study, the GTHE approach was developed based on several classical methods and then modified according to the potential influencing factors step by step to fit the contaminated soil samples. After the optimization of the GTHE approach, high RNA recovery rate ($4.10 \sim 59.41$ μg g^{-1}) from all the soil samples was obtained, along with high-quality RNA for qRT-PCR and metatranscriptomic analyses.

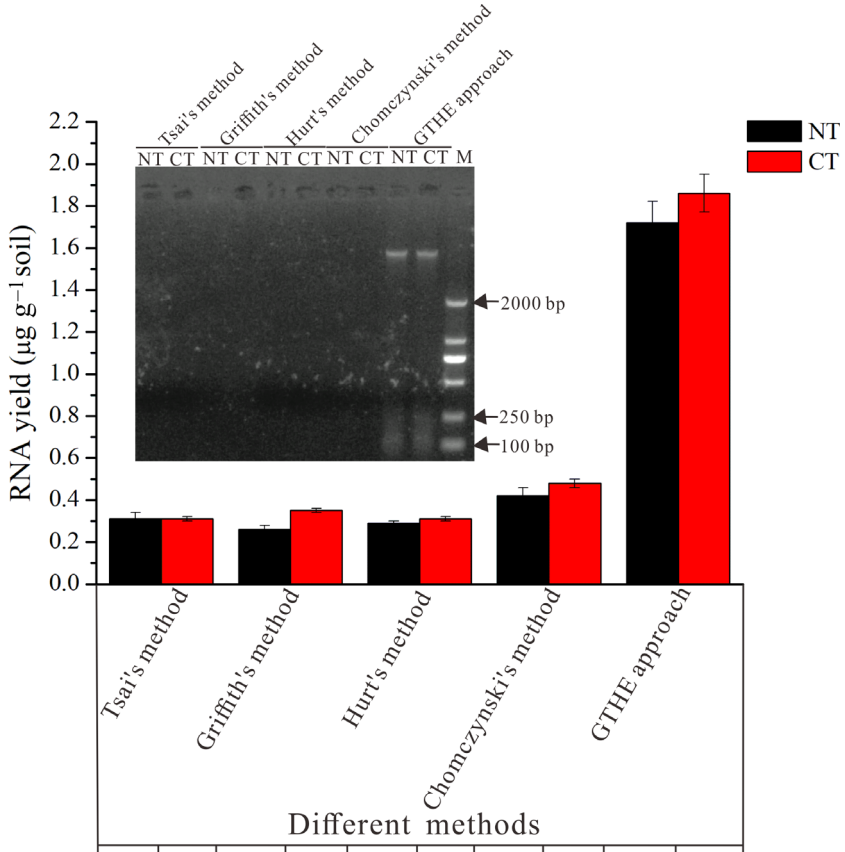
Results and discussion

Comparison of classical methods and the GTHE approach on the severely heavy metal-contaminated soil samples

To investigate the soil total microbial RNA extraction efficiency on NT and CT, four classical methods including Tsai's method (Tsai *et al*., 1991; Daniell *et al*., 2012), Griffith's method (Griffiths *et al*., 2000; Epelde *et al*., 2015), Hurt's method (Hurt *et al*., 2001; Rittmann and Holubar, 2014), Chomczynski's method (Chomczynski and Sacchi, 1987; Kambura *et al*., 2016), along with the GTHE approach developed based on these representative methods, were used in this study. NT and CT samples were collected from a long-term industrial contaminated site that has been previously reported to be severely contaminated with heavy metals (Zhang, 2013; Huang *et al*., 2017). According to the Environmental Quality Standards for Soils of China (GB15618-1995), the concentrations of Zn ($341.96 \sim 342.55$ mg kg^{-1}) and Ni ($73.45 \sim 75.57$ mg kg^{-1}) in the samples were considered under moderate level contamination, while Cr ($506.58 \sim 563.98$ mg kg^{-1}) and Cd ($2.59 \sim 2.60$ mg kg^{-1}) were classified under high-level contamination (Table 2). The GTHE approach efficiently extracted $1.72 \sim 1.86$ μg RNA per gram of soil, while the RNA extracted by other four classical

methods displayed nothing on gel (Fig. 1). This result indicated that GTHE approach was an efficient method for RNA extraction from soils with heavy metal pollution.

In this study, microbial biomass, organic pollutants, heavy metals, and RNAase are considered as the potential influencing factors based on the physiochemical properties of NT and CT. Although the classical methods resulted in sufficient RNA from common natural soil samples, they could not acquire enough RNA from NT or CT samples. The prokaryotic biomass of NT and CT samples gave a low yield of $\sim 5.5 \times 10^6$ 16S rDNA copies per gram of soil because of severe pollution (Fig. S2), which is lower than the microbial amount reported in common soils, ranging from $10^8 \sim 10^{10}$ 16S rDNA copies per gram of soil (Soule *et al.*, 2009; Semedo *et al.*, 2018). The intense cracking procedures employing multiple denaturants and mechanical lysis technique were performed simultaneously in the original GTHE approach (Table 1). These intense cracking procedures did not affect RNA quality (Liu *et al.*, 2011), but resulted in more cell disruption and the release of RNA (Wang *et al.*, 2009). The higher RNA yield by GTHE approach is in accordance with a previous study that applied mechanical homogenization in Trizol for releasing microbial RNA (Liu *et al.*, 2011). Trizol is a monophasic solution of phenol and guanidinium thiocyanate, which have also been included in Tsai's method (Tsai *et al.*, 1991; Daniell *et al.*, 2012), Chomczynski's method (Chomczynski and Sacchi, 1987; Kambura *et al.*, 2016), and the GTHE approach. In addition, the anion-exchange resin column (Qiagen, Hilden, Germany), based on the interaction between negatively charged phosphates of the RNA and positively charged diethylaminoethyl (DEAE) groups on the surface of the resin,



	NT	CT	NT	CT	NT	CT	NT	CT	NT	CT
Yield ($\mu g\ g^{-1}$ soil)	0.31 ±0.03	0.31 ±0.01	0.26 ±0.02	0.35 ±0.01	0.29 ±0.01	0.31 ±0.01	0.42 ±0.04	0.48 ±0.02	1.72 ±0.10	1.86 ±0.09
A260/A280	1.46 ±0.08	1.58 ±0.03	1.55 ±0.11	1.46 ±0.05	1.72 ±0.02	1.42 ±0.05	1.46 ±0.05	1.69 ±0.02	2.03 ±0.00	2.03 ±0.02
A260/A230	0.46 ±0.04	0.49 ±0.09	0.54 ±0.07	0.48 ±0.04	0.44 ±0.03	0.55 ±0.02	0.52 ±0.01	0.59 ±0.03	1.56 ±0.04	1.43 ±0.02

Fig. 1. Quality and quantity of the total microbial RNA extracted from NT and CT samples using five different methods, including Tsai's method, Griffith's method, Hurt's method, Chomczynski's method, and GTHE approach.

Table 1. Summary of different soil total microbial RNA extraction methods.

Methods[a]	Lysis procedures	Purification procedures	Time	Reference
Tsai's method	Guanidine thiocyanate, 2-mercaptoethanol, phenol: chloroform: isoamyl alcohol (25:24:1)	Cold isopropanol precipitation	3 h	Tsai *et al.* (1991), Daniell *et al.* (2012)
Griffith's method	CTAB, phenol: chloroform: isoamyl alcohol; ceramic beads (ZrO_2 64% and SiO_2 33%) beating for 30 s	Polyethylene glycol 6000	3 h	Griffiths *et al.* (2000), Epelde *et al.* (2015)
Hurt's method	CTAB, SDS, phenol: chloroform: isoamyl alcohol (25:24:1); samples mixed with sand and grounded in liquid nitrogen	Isopropanol precipitation	2.5 h	Hurt *et al.* (2001), Rittmann and Holubar (2014)
Chomczynski's method	Guanidine thiocyanate, 2-mercaptoethanol phenol, chloroform: isoamyl alcohol (49:1), shake 10 s	Isopropanol precipitation	3.5 h	Chomczynski and Sacchi (1987), Kambura *et al.* (2016)
GTHE approach	Guanidine thiocyanate, CTAB, SDS, phenol: chloroform: isoamyl alcohol (25:24:1); silica carbide beads beating for 15 min	Isopropanol precipitation and the anion-exchange resin column (Qiagen)	2.5 h	This study

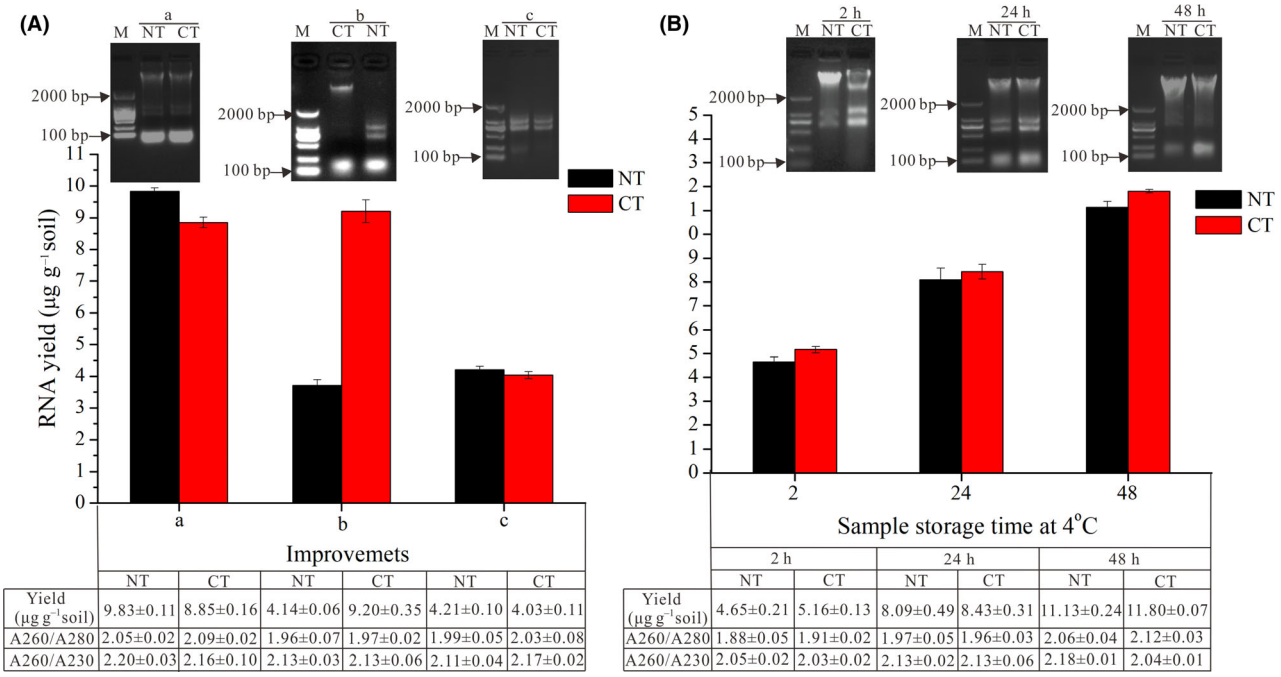
a. The method was named after their first author of the source reference.

was used in GTHE approach to ensure further removal of contaminants from the RNA. Although heavy metals and organic pollutants usually co-exist, the influence of organic pollutants was not deeply observed in this study due to the following reasons: (i) organic reagents used in the process of RNA extraction removed the majority of the organic pollutants; (ii) the residue organic pollutants were eliminated from RNA by isopropanol precipitation and the further anion-exchange resin column (Qiagen) purification in the GTHE approach. Besides, the total microbial RNA extraction process by this GTHE approach took 2.5 h, which is much less than the conventional methods (Table 1). Therefore, the GTHE approach becomes more efficient than other methods by combining the advantages of the classical methods. And it could be considered as a promising method for the total microbial RNA extraction from contaminated soils.

Optimization of the GTHE approach

The impact of soil amount (microbial biomass), RNAase, and heavy metals on RNA extraction efficiency was reduced by optimizing the GTHE approach. Increasing initial soil amount from 2 g to 4 g and doubling the proportion of chemical reagents simultaneously, raised the RNA yield, despite of severe degradation (Fig. 2A). Since the initial soil amount was doubled, more endogenous and exogenous RNAase was introduced into the system. Low temperature not only promotes the flocculation of nucleic acids and thus forming more pellets under centrifugal forces, but also inhibits the activity of RNAase to protect RNA from degradation (Sheng *et al.*, 2005). Therefore, the whole extraction process was further kept at 4°C to increase RNA yield and alleviate severe RNA degradation. After this modification, although the RNA yield did not increase, the integrity of RNA in NT group exhibited better integrity than before. In addition to 4°C treatment, a classical method called Hurt's method was performed at room temperature (RT) (Hurt *et al.*, 2001; Rittmann and Holubar, 2014). Therefore, to analyse the effect of temperature on RNA extraction process, the GTHE approach was first developed at RT. In contrast to NT, the total microbial RNA of CT still presented severe degradation (Fig. 2A). This result indicated that Cr (VI) has a great influence on RNA integrity. Due to the degradation, the detected concentration of total RNA in CT was greater than NT. RNA degradation of CT group is consistent with the previous, which reported that exhibited RNA extraction from Cr(VI) contaminated soils as challenging (Pradhan *et al.*, 2016). It is probably due to the high risk of RNA degradation caused by the formed ROS in the process of intracellular chromate reduction (Pradhan *et al.*, 2016). Besides, the high-quality and high-yield total microbial RNA could be extracted from an uncontaminated flowerpot soil sample (Fig. S3), which further indicated that heavy metal contamination is one of the main factors influencing the RNA recovery efficiency. Additionally, a certain degree of RNA degradation in NT group also indicated that heavy metals or RNAase still has a great influence on RNA despite the above modifications.

Guanidine thiocyanate is a strong protein denaturant. It not only strongly inhibits the activity of RNase, but also effectively dissociates the complex of protein and nucleic acid, thereby enhancing the quality of RNA extraction (Liu *et al.*, 2011). To isolate more intact RNA from NT and CT samples, RNAase was further excluded by increasing 3% guanidine thiocyanate from 2 ml to 2.5 ml in step I based on RNA degradation level as shown in Figure 2A. EDTA is responsible for chelating heavy metals (McDougall *et al.*, 2019). To eliminate the influence of heavy metals on RNA integrity, the concentration of EDTA was raised from 10 mM to 100 mM in steps I and III according to the amount used in Hurt's method (Hurt *et al.*, 2001; Rittmann and Holubar, 2014). After the adjustments, RNA yield, A260/A280, and A260/A230



	Improvemets					
	NT	CT	NT	CT	NT	CT
Yield (μg g^{-1}soil)	9.83±0.11	8.85±0.16	4.14±0.06	9.20±0.35	4.21±0.10	4.03±0.11
A260/A280	2.05±0.02	2.09±0.02	1.96±0.07	1.97±0.02	1.99±0.05	2.03±0.08
A260/A230	2.20±0.03	2.16±0.10	2.13±0.03	2.13±0.06	2.11±0.04	2.17±0.02

	2 h		24 h		48 h	
	NT	CT	NT	CT	NT	CT
Yield (μg g^{-1}soil)	4.65±0.21	5.16±0.13	8.09±0.49	8.43±0.31	11.13±0.24	11.80±0.07
A260/A280	1.88±0.05	1.91±0.02	1.97±0.05	1.96±0.03	2.06±0.04	2.12±0.03
A260/A230	2.05±0.02	2.03±0.02	2.13±0.02	2.13±0.06	2.18±0.01	2.04±0.01

Fig. 2. Quality and quantity of the total microbial RNA extracted from NT and CT samples (A) with different improved conditions, including (a) increasing initial soil amount and proportion of subsequent chemical reagents, (b) increasing initial soil amount and proportion of subsequent chemical reagents and keeping at 4°C during the whole extraction process, (c) increasing initial soil amount and proportion of subsequent chemical reagents, keeping at 4°C during the whole extraction process, and the adjustment of the chemical reagents (increasing 3% guanidine thiocyanate from 2 ml to 2.5 ml and EDTA from 10 mM to 100 mM); (B) with different storage times at 4°C.

from NT and CT samples ranged 4.03 ~ 4.21 μg g^{-1}, 1.99 ~ 2.03, and 2.11 ~ 2.17 (Fig. 2A), respectively. The RNA yield is much higher than the previously reported result of the mining soil sample, which gave up to 0.22 ~ 0.5 μg RNA per gram of soil (Lehembre *et al.*, 2013). This indicated the high yield, integrity, and purity of total RNA extracted from the severely heavy metal-contaminated soil by the GTHE approach after optimization.

In addition to the above influencing factors, storage ways of NT and CT samples were also examined in this study. Previous studies showed that high-quality RNA could be successfully extracted from soil samples that were collected and immediately kept at −80°C or liquid nitrogen (Sessitsch *et al.*, 2002; Mcgrath *et al.*, 2008). Our results are in contrast to the previous studies that showed that a high-quality RNA was isolated from soils stored at 4°C, whereas the samples stored under −80°C or liquid nitrogen presented severe RNA degradation (data not shown). This might be due to the reason that cell structure is easily broken after freezing and thawing, especially for high water content samples, which results in the release of more endogenous RNAase. Thus, RNA presented more severe degradation here (Jun *et al.*, 2018). Several studies have reported to store the samples at −80°C or in liquid nitrogen. However, the lysis procedures are performed under −80°C rather than 4°C or room temperature, which results in high-quality RNA isolation (Hurt *et al.*, 2001; Epelde *et al.*, 2015; Tournier *et al.*, 2015).

Further, the quality of the total microbial RNA extracted from soil samples stored at 4°C with different times was also explored. The results showed that fresh soil sample (2 h) is the best for yielding high-quality RNA, while the RNA extracted from 24 h and 48 h stored soil samples presented moderate and severe degradation, respectively (Fig. 2B). Because of the higher RNA degradation rate of samples stored at 24 h and 48 h, the detected concentrations of the total RNA also increased. This result suggested that it is better to extract RNA from a fresh soil sample. In addition, previous studies reported that storage at 4°C for a short time will not impact the microbial biomass and activities (Barros *et al.*, 2017). Combined with the relatively higher RNA integrity yielded from NT and CT in 24 h (Fig. 2B), we suggested that the soil samples need to be stored for no more than 24 h at 4°C. RNALater can kill the microbes, maintain the in situ metabolism of microorganisms, and lower down the degradation rate of RNA in microbes, which is widely used to store samples for RNA analysis (Schnecker *et al.*, 2012). To prevent the huge shift of in situ microbial metabolism in longer storage time, samples can be preserved in RNALater

(Rissanen *et al.*, 2010). After all adjustments, the extracted total microbial RNA was resuspended and stored in the RNA Storage Solution (Ambion, Carlsbad, CA, USA) instead of RNAase-free water to ensure RNA's stability under −80°C. After that, gDNA was removed by the DNAase supplied in PrimeScript RT reagent kit (Takara, Tokyo, Japan). Finally, the optimized GTHE method is presented in Figure 3.

Application of the GTHE method on various types of heavy metal-contaminated soil samples

To further validate the application potential of the GTHE method, it was applied to different types of soils including NT, CT, grassland soil, rhizosphere soil, farmland soil, and forest soil. Activated sludge, which is used for wastewater treatment and is mostly contaminated by heavy metals (Yu and Zhang, 2012), was also utilized to stimulate the high microbial biomass contained soil. The physicochemical properties of the above environmental samples are presented in Table 2. All the samples showed diverse moisture content (8.98 ~ 78.75%). The concentration of TC varied from 33.26 to 167.50 mg g^{-1}, while the TOC content ranged from 18.11 to 98.06 mg g^{-1}. Activated sludge was the largest N-contained sample, reaching up to 26.60 mg g^{-1}, followed by NT and CT samples, whereas other samples possessed less N. The average pH of these samples varied from 6.92

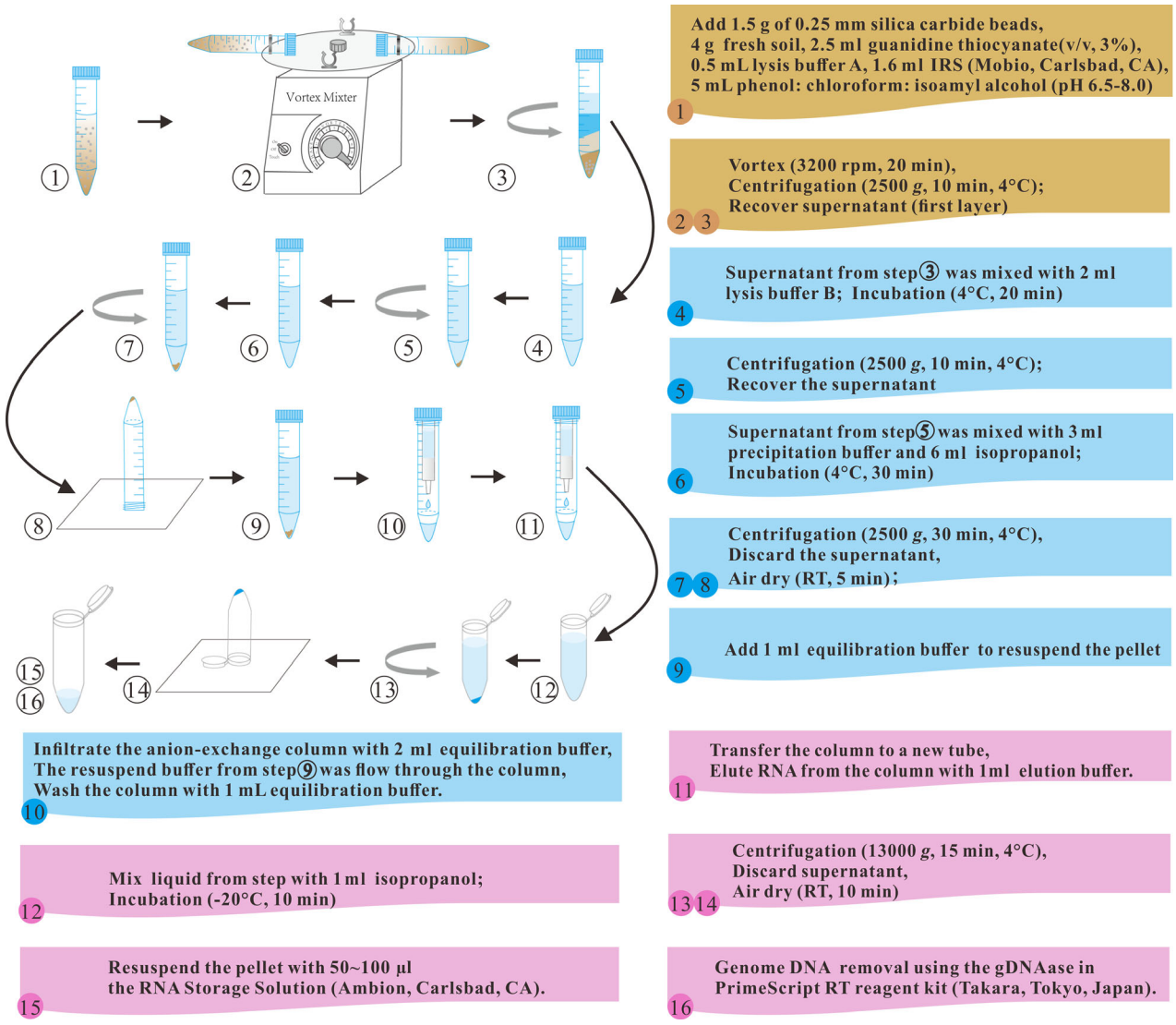


Fig. 3. Procedures for the optimized GTHE method. Lysis buffer A (100 mM sodium phosphate (pH 7.0), 100 mM Tris–HCl (pH 7.0), 5% CTAB, 20% SDS, 150 mM NaCl, and 100 mM EDTA); lysis buffer B (100 mM Tris–HCl (pH 7.0), 20% SDS, 150 mM NaCl, and 100 mM EDTA), precipitation buffer (100 mM Tris–HCl (pH 7.0) and 5 M NaCl), equilibration buffer (20% isopropanol and 150 mM NaCl), elution buffer (20% isopropanol and 3 M NaCl).

Table 2. Physicochemical characteristics of different types of heavy metal-contaminated soil samples.

Physicochemical properties	Riparian soil[a]		Grassland soil	Rhizospheres soil	Farmland soil	Forest soil	Activated sludge
	NT (Pei *et al.*, 2018)	CT					
pH	7.98 ± 0.06	7.93 ± 0.05	7.20 ± 0.03	7.25 ± 0.03	7.13 ± 0.02	7.16 ± 0.03	6.92 ± 0.03
Moisture (%)	37.61 ± 3.88	39.27 ± 1.16	34.17 ± 0.01	17.17 ± 0.02	8.98 ± 0.05	18.10 ± 1.77	78.75 ± 1.27
TN (mg g^{-1})	14.40 ± 1.53	15.04 ± 0.73	1.27 ± 0.09	3.33 ± 0.18	1.17 ± 0.34	1.27 ± 0.43	26.60 ± 3.53
TC (mg g^{-1})	56.81 ± 2.70	56.00 ± 4.89	41.18 ± 1.02	66.64 ± 1.72	33.26 ± 0.74	39.26 ± 1.33	167.50 ± 19.27
TOC (mg g^{-1})	29.57 ± 6.13	28.28 ± 0.51	21.95 ± 0.79	35.58 ± 1.39	18.11 ± 0.29	20.94 ± 0.33	98.06 ± 3.26
K (mg g^{-1})	18.22 ± 2.59	18.45 ± 1.25	16.54 ± 0.67	15.92 ± 0.95	26.73 ± 1.88	43.93 ± 1.95	15.65 ± 0.77
Na (mg g^{-1})	46.26 ± 1.91	45.86 ± 1.61	37.05 ± 3.17	44.80 ± 2.46	55.29 ± 4.68	57.53 ± 1.96	26.30 ± 3.59
Cu (mg kg^{-1})	28.58 ± 3.83	27.87 ± 1.13	56.54 ± 0.45 (I)	37.20 ± 1.56 (I)	34.88 ± 6.73	29.75 ± 1.08	61.78 ± 6.05 (I)
Zn (mg kg^{-1})	342.55 ± 4.68 (II)	341.96 ± 15.29 (II)	186.27 ± 12.62 (I)	127.23 ± 2.74 (I)	141.68 ± 6.05 (I)	121.04 ± 7.74 (I)	283.48 ± 5.01 (II)
Ni (mg kg^{-1})	73.45 ± 5.99 (II)	75.57 ± 5.55 (II)	62.21 ± 0.66 (II)	84.80 ± 4.44 (II)	65.63 ± 2.15 (II)	66.12 ± 2.28 (II)	67.26 ± 3.62 (II)
Mg (mg kg^{-1})	1327.15 ± 50.83	1378.71 ± 39.54	5352.77 ± 298.83	3800.81 ± 330.48	2578.36 ± 170.48	3189.24 ± 281.30	3219.26 ± 17.81
Al (mg kg^{-1})	13939.33 ± 725.25	14535.80 ± 663.66	10250.45 ± 739.10	14627.94 ± 1350.03	18492.72 ± 645.26	9109.54 ± 614.14	10726.43 ± 205.31
Fe (mg kg^{-1})	35035.41 ± 1581.89	36415.25 ± 690.75	23605.14 ± 214.42	22964.79 ± 600.38	23846.53 ± 553.86	23445.38 ± 868.74	20895.95 ± 279.27
Mn (mg kg^{-1})	10753.33 ± 45.09	11074.33 ± 518.32	512.27 ± 2.19	496.67 ± 15.71	527.50 ± 12.86	512.10 ± 11.51	336.44 ± 3.81
Cr (mg kg^{-1})	506.58 ± 13.79 (III)	563.98 ± 1.69 (III)	163.05 ± 1.42	166.20 ± 3.09	169.12 ± 4.75	167.54 ± 3.90	173.83 ± 4.86
Pb (mg kg^{-1})	17.21 ± 3.50	19.73 ± 0.86	45.56 ± 0.99 (I)	39.17 ± 4.01 (I)	35.29 ± 3.37 (I)	33.69 ± 2.86	25.07 ± 4.16
Cd (mg kg^{-1})	2.59 ± 0.10 (III)	2.60 ± 0.12 (III)	2.62 ± 0.10 (III)	2.75 ± 0.33 (III)	8.43 ± 0.60 (III)	2.53 ± 0.41 (III)	2.44 ± 0.10 (III)
As (mg kg^{-1})	13.41 ± 2.96	13.56 ± 2.24	7.19 ± 1.55	6.39 ± 2.71	213.20 ± 6.05 (III)	16.70 ± 1.57 (I)	26.95 ± 2.69 (I)

a. The riparian soil was divided into NT and CT samples, which were treated with 0.85% NaCl solution only and 0.85% NaCl solution containing 1 mM Cr (VI), respectively. mM Cr (VI), respectively.

to 7.98. According to the Environmental Quality Standards for Soils of China (GB15618-1995) concentrations of heavy metals are divided into three levels (I, II, and III, corresponding to slightly polluted, moderately polluted, and heavily polluted, respectively). The concentrations of Ni and Cd in all samples were classified under levels II and III separately, whereas the concentration of Zn was classified as level I or II. The concentration of Cu in grassland soil, rhizosphere soil, and activated sludge was higher than the background limit, while the concentration of Pb in grassland, rhizospheres, and farmland soils also exceeded the background limit. NT and CT samples were polluted severely by Cr, which was classified as level III. The concentration of arsenic (As) in farmland soil sample was considerably higher (up to 10.66-times higher) than the natural background (15 mg kg^{-1}), whereas the forest soil and activated sludge samples were moderately polluted by As (Table 2).

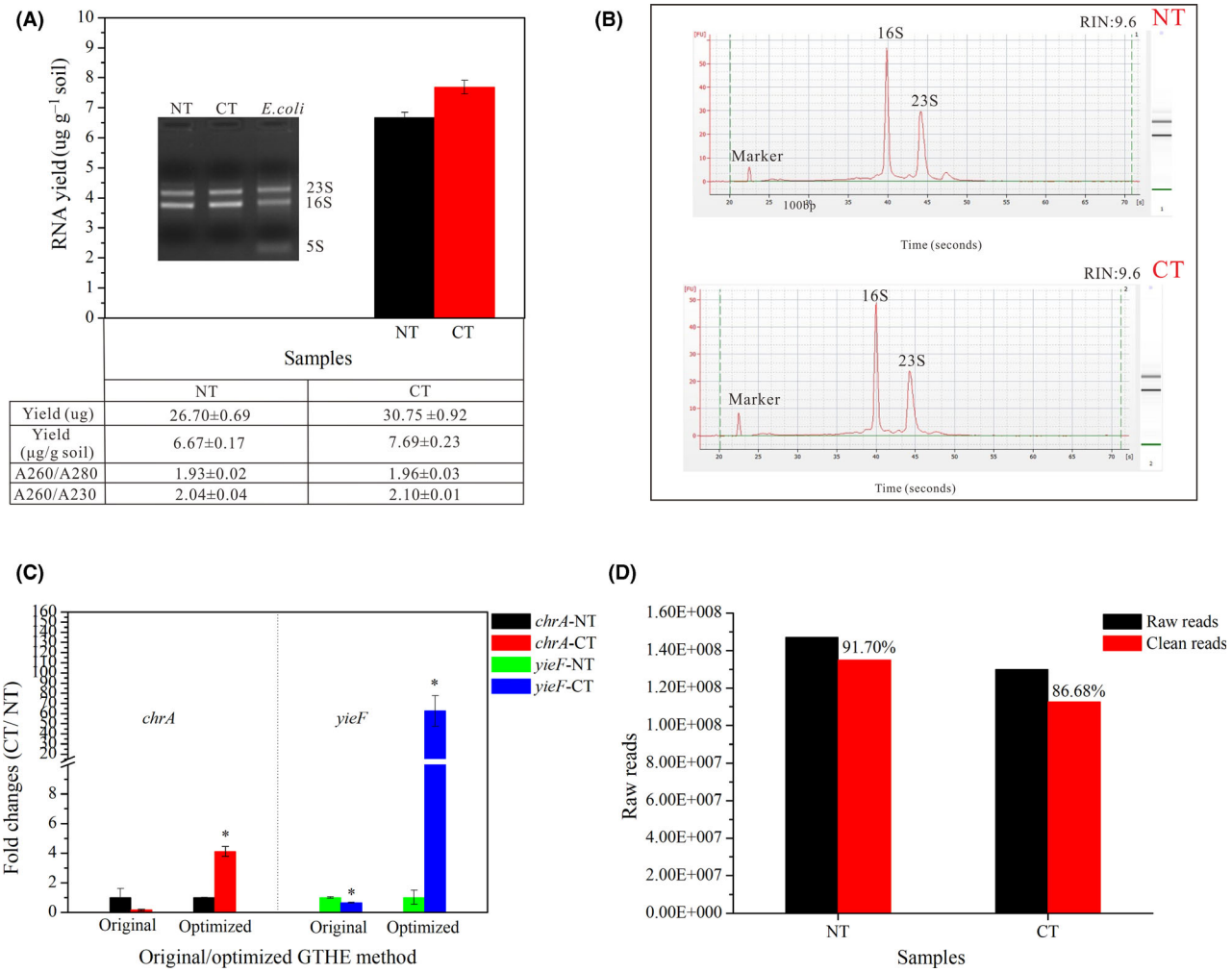
The severe heavy metals (Zn, Ni, Cr, and Cd) contamination in CT and NT samples originated from the upstream industrial companies as previously described (Pei *et al*., 2018). The contaminations of Ni and Cd in all samples were classified as under levels II and III, respectively (Table 2), which may be caused by the perennial discharge and sedimentation of Ni/Cd-containing exhaust gas of the manufactories in Lanzhou (Gao *et al*., 2004; Liu *et al*., 2009). The concentration of As in farmland was 213.20 mg kg^{-1} (Table 2). Phenylarsonic additives, such as p-arsanilic acid and roxarsone, are often used during animal feeding operations to promote growth and prevent disease in aquaculture (Chen *et al*., 2017). The As compounds and their metabolites in animal waste are always released into farmland as fertilizer (Shi *et al*., 2018). In addition, As is also often found in some pesticides, disinfectants, fungicides, and herbicides, which may be other pollution sources (Chen *et al*., 2017; Shi *et al*., 2018). As for grassland, rhizosphere, farmland, and forest soils, due to the frequent anthropic activity, are very likely to be the combined polluted sites.

The texture of soils is shown in Figure S4 and classified as loam or loamy sand, with clay contents, ranging slightly from 5.55 to 9.04% (Table S1). Despite using varying sample amounts from all type of soils, RNA extracted by the GTHE method and exhibited high yield and high quality (Table S1 and Fig. S5). In this study, the RNA recovery efficiency varied from 2.80 to 59.41 $\mu g\ g^{-1}$ soil as detected by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Thus, much higher cDNA amount can be yielded after performing reverse transcription. The efficiency is much higher than the summary in a previous study reported that $2 \sim 5$ μg cDNA amount for high-throughput sequencing needs to be recovered from $10 \sim 100$ g soil samples, depending on the soil types and RNA extraction methods (Wang *et al*., 2009). The abundance levels in grassland soil and rhizospheres soil were 8.58×10^7 and 7.05×10^7 16S rDNA gene copies per gram soil, respectively (Fig. S3), which is significantly higher than the other five soil samples. This result indicated that these two samples contained more prokaryotic biomass than other samples. RNA yields of grassland soil and rhizospheres soil are the second and third highest, which indicated that these two samples also contained more active microbes. The activated sludge showed the highest RNA concentration, but the lowest 16S rDNA copy number. This may be due to the reason that the RNA amount represents all the active microbes in the environment, while the 16S rDNA copy number only reflects the number of prokaryotic microorganisms in activated sludge. In accordance with the present results, a previous study demonstrated that activated sludge is a different microbial system compared with soils, containing large amounts of active eukaryotes, bacteria, archaea, and viruses for wastewater treatment (Yu and Zhang, 2012).

RNA application on comprehensive molecular analyses

The isolation of high-quality RNA is necessary for qRT-PCR and metatranscriptome analysis of severely heavy metal-contaminated samples. Cr is one of the most common heavy metals found in contaminated soils. The mechanisms of Cr(VI) remediation genes are relatively clear (Pradhan *et al*., 2016). NT and CT samples were collected from a severely Cr contaminated site, where microbes are most likely to contain Cr (VI) remediation genes. NT sample was treated with 0.85% NaCl, while CT sample was treated with 0.85% NaCl containing 1 mM Cr (VI) for 30 min. RNA of these two representative samples was then extracted and further qualified by qRT-PCR and metatranscriptomic sequencing. This independent repeat experiment showed similar results as previous results in this study. The 23S, 16S, and 5S rRNA bands of total microbial RNA were clearly distinct on agarose gel electrophoresis. Totally, 26.70 μg and 30.75 μg total microbial RNA were yielded from NT and CT samples, respectively. Ratios of A260/A280 and A260/A230 ranged from 1.93 to 1.96 and 2.04 to 2.10, respectively (Fig. 4A), suggesting the high purity of the extracted total microbial RNA from heavy metal-contaminated soils. This result also indicated the GTHE method is repeatable. Besides, the result of the Agilent 2100 Bioanalyzer showed that RNA integrity number (RIN) in both NT and CT samples reached 9.6 (maximum number: 10) (Fig. 4B), which further illustrated the high integrity of total microbial RNA.

The expression fold changes of two common chromate remediation genes *chrA* and *yieF* were examined by qRT-PCR with RNA extracted by different methods. The results of qRT-PCR performed with RNA isolated by



	NT	CT
Yield (ug)	26.70±0.69	30.75 ±0.92
Yield (μg/g soil)	6.67±0.17	7.69±0.23
A260/A280	1.93±0.02	1.96±0.03
A260/A230	2.04±0.04	2.10±0.01

Fig. 4. (A) Quality and quantity of the total microbial RNA extracted from NT and CT samples by the GTHE method. (B) Integrity of soil total microbial RNA was identified by Agilent 2100. RNA integrity number (RIN) values range from 1 (most degraded) to 10 (most intact). Fluorescence and time are equivalent to the abundance and the size of RNA fragments, respectively. (C) Expression levels of *chrA* and *yieF* detected with RNA extracted by the original and optimized GTHE method. Mean expression in the CT group is shown as fold-change compared with the mean expression in the NT group, which has been ascribed an arbitrary value of 1. Significant difference at the $P < 0.05$ level is signified by * as determined by Tukey's test. (D) Raw reads and clean reads of NT and CT in metatranscriptomic libraries.

the optimized GTHE method showed that the expressions of *chrA* and *yieF* in CT compared with NT were upregulated 4.12- and 62.43-fold, respectively (Fig. 4C). This is consistent with previous studies that observed an increase in the expression levels of *chrA* and *yieF* after Cr(VI) treatment in pure bacteria or cells (Flores-Alvarez *et al.*, 2012; Liu *et al.*, 2015). However, due to the severe degradation of RNA extracted by the original GTHE approach, the expressions of *chrA* and *yieF* were very low, and even showed downregulation after Cr(VI) treatment (Fig. 4C). In addition, because of the low yield and possible degradation of RNA isolated by Tsai's, Griffith's, Hurt's, and Chomczynski's methods, *chrA* and *yieF* were undetectable in these RNA samples.

The total microbial RNA extracted from NT and CT samples reached to 26.70 μg and 30.75 μg, respectively, which are much higher than the required amount (5 μg) of Ribo-Zero Magnetic Kit (Epicenter, Madison, WI) for metatranscriptomic libraries construction. Therefore, Ovation® RNA-Seq System V2 Kit, which is specialized on samples of low amount RNA (Song *et al.*, 2018), has not been applied in our study. The metatranscriptomic sequencing result showed that 134,842,286 (91.70%) and 112,635,252 (86.68%) clean reads of mRNA were obtained in NT and CT groups, respectively (Fig. 4E). The high ratios of mRNA read in total RNA datasets of NT and CT indicated that the high-quality libraries were constructed for metatranscriptomic

analysis. These data implied that the microbial total RNA extracted from the severely heavy metal-contaminated soil samples by the GTHE method can be applied for qRT-PCR and metatranscriptomic analyses. This method thus will facilitate us to comprehensively explore and understand more heavy metal-contaminated territories.

This study provided an effective and robust method of recovering high quantity and quality of RNA from seriously heavy metal-contaminated soils for downstream comprehensive molecular analyses. Although high-quality RNA from different soils can be isolated by the GTHE method, the initial soil amount needs to be explored based on their physicochemical properties. It is also important to reduce the effect of possible influencing factors on RNA recovery efficiency. Further, this method cannot be guaranteed to be applied to all types of soils. However, known factors that influence RNA extraction efficiency, including microbial biomass, heavy metals, RNAase, sample storage ways, and times, and the corresponding solving strategies provide an important reference to other soil total microbial RNA extraction methods. Therefore, this study will facilitate researchers to figure out the ecological role of active microbes in severely heavy metal-contaminated soil habitats and further contribute to in situ heavy metal remediation by microbes.

Experimental procedures

Sample collection and characterization

Samples were collected to validate and optimize the GTHE method. The riparian soil sample was collected from a small tributary of the Yellow River where numerous industrial factories were located upstream. This soil sample was divided into two subsamples: one treated with 0.85% NaCl was defined as NT sample, while another treated with 0.85% NaCl containing 1 mM Cr (VI) for 30 min was CT sample. In our previous studies, we found the site to be heavily contaminated with heavy metals (such as Zn and Cr) and polycyclic aromatic hydrocarbons (PAHs) (Yu *et al*., 2016; Huang *et al*., 2017; Pei *et al*., 2018). Flowerpot soil was taken from a flowerpot, which is cultured by the uncontaminated commercial soil. Grassland soil, farmland soil, and forest soil were sampled from long-term grassland, vegetable cultivation, and trees land, respectively. Rhizosphere soil sample was collected from the rhizosphere of a laboratory-raised plant, whose cultivated soils were originally collected on campus. Activated sludge used for wastewater treatment is usually accompanied by heavy metals (Miao *et al*., 2015) and a large number of microbes (Yu and Zhang, 2012). Therefore, it was used to simulate heavy metal-contaminated soil samples with high microbial biomass in this study. The activated sludge sample was collected from an urban sewage treatment plant, which is contaminated with alcohol ethoxylate (Ji *et al*., 2019). Each sample composed of five combined subsamples that were taken from 10 cm depth as we described before and immediately transferred to the lab on ice within an hour. An aliquot of each soil sample was kept and stored at 4°C, while the remainder was used for soil characterization. Briefly, soil moisture was measured by the thermo-gravimetric method (Al-Kayssi, 2002). One part of soil samples was homogenized and sieved via 100-mesh sieve to determine pH, total nitrogen (TN), total carbon (TC), total organic carbon (TOC), and metals. The other part of soil samples recovered using a 10-mesh sieve was used to determine the particle size composition, which was carried out with a laser diffraction instrument (Malvern Mastersizer 2000, Worcestershire, UK) (Houghton *et al*., 2002). Incubation with 1 M KCL for two hours was used to determine soil pH (Wu *et al*., 2010). TC, TOC, and TN contents were determined using a Costech Elemental Analyzer (Costech Analytical Technologies Inc., Valencia, CA, USA) (Prober *et al*., 2015). The samples were digested to detect the total concentration of metals (K, Na, Cu, Zn, Ni, Mg, Al, Fe, Mn, Cr, Pb, Cd, and As) by flame atomic absorption spectrometry (FAAS) (Analytik Jena, Berlin, Germany) as previously described (Tao *et al*., 2019).

Instructions of five soil total microbial RNA extraction methods

Four classical RNA extraction methods, including Tsia's method (Tsai *et al*., 1991; Daniell *et al*., 2012), Griffith's method (Griffiths *et al*., 2000; Epelde *et al*., 2015), Hurt's method (Hurt *et al*., 2001; Rittmann and Holubar, 2014), Chomczynski's method (Chomczynski and Sacchi, 1987; Kambura *et al*., 2016), and the GTHE approach established based on the above classical methods, were evaluated on the contaminated soils in this study. Soil total microbial RNA was extracted according to the instructions of aforementioned methods. All reagents were prepared with 0.1% diethyl pyrocarbonate (DEPC) water. The steps of the original GTHE approach are as follows:

i. 2 g of fresh soil, 1.5 g of 0.25 mm silica carbide beads, 1 ml of 3% guanidine thiocyanate, 0.25 ml of lysis buffer A (100 mM sodium phosphate (pH 7.0), 100 mM Tris–HCl (pH 7.0), 5% CTAB, 20% SDS, 150 mM NaCl, and 10 mM EDTA), 0.8 ml of inhibitor removal solution (IRS) (Mobio, Carlsbad, CA, USA), and 2.5 ml of phenol: chloroform: isoamyl alcohol (pH 6.5–8.0) were added to the 15-ml tube in order;
ii. The mixture was vortexed at 3200 r.p.m. for 20 min and centrifugated at 2500 × *g* for 10 min at RT;

iii. The top aqueous phase was transferred to a clean 15-ml tube; 1 ml of lysis buffer B (100 mM Tris–HCl (pH 7.0), 20% SDS, 150 mM NaCl, and 10 mM EDTA) was added; and then, the tube was vortexed, incubated at RT for 20 min, and centrifuged at 2500 × g for 10 min at RT;
iv. 1.5 ml of precipitation buffer (100 mM Tris–HCl (pH 7.0) and 5 M NaCl) was added to supernatant with 3 ml isopropanol and then vortexed, incubated at RT for 30 min, centrifuged at 2500 × g for 30 min at RT;
v. The pellet was air-dried for 5 min, and then, 1 ml of equilibration buffer (20% isopropanol and 150 mM NaCl) was used to resuspend the pellet;
vi. 2 ml of equilibration buffer was used to infiltrate the anion exchange resin column (Qiagen), and buffer from step (v) was added to the column. The column was subsequently washed with 2 ml of equilibration buffer, and the RNA was eluted from the column using 1 ml of elution buffer (20% isopropanol and 3 M NaCl);
vii. The eluted RNA was precipitated with 1 ml of isopropanol, mixed, incubated at −20°C for at least 10 min, and then centrifuged at 13 000 × *g* for 15 min at 4°C;
viii. The pellet was air-dried for 5 min and resuspended in 50 ~ 100 μl of RNase-free water.

Three replicates of each NT and CT set were mixed as one sample for RNA quality evaluation by 1.0% agarose gel electrophoresis, qRT-PCR and meta-analysis.

Improvement of the GTHE approach

To exclude the known factors that influence RNA extraction efficiency of NT and CT samples, the procedures of the GTHE approach were optimized. The increasing initial soil amount was increased from 2 g to 4 g, and the doubled proportion of subsequent chemical reagents was doubled. Keeping the whole RNA extraction process at 4°C, the adjustment of the chemical reagents (increasing 3% guanidine thiocyanate from 0.5 ml g^{-1} soil to 0.625 ml g^{-1} soil and raising 10 mM from EDTA to 100 mM) was done. Besides, storage ways and times of NT and CT samples were also examined with the GTHE method.

RNA quality evaluation

The total microbial RNA was quantified with 1.0% agarose gel electrophoresis and Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA). RNA sample for meta-sequencing was also evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) before sequencing. RNA with the standard of A260/A280 = 1.8 ~ 2.2, A260/A230 ≥ 2.0, 23S:16S ≥ 1.0, RIN ≥ 6.5, and > 10 μg was regarded as the high-quality RNA, which was pre-reserved and used for meta-sequencing analysis (Zhou *et al.*, 2017).

Real-time quantitative PCR (qPCR) and quantitative reverse transcription-PCR (qRT-PCR)

The total microbial RNA extracted by different methods from both NT and CT samples was reverse-transcribed as cDNA. Then, the expression levels of two tested genes, *yieF* (chromate reductase) and *chrA* (chromate transporter) in both groups were determined by qRT-PCR. Primer sequences and reactions were previously detailed (Pei *et al.*, 2018). The expression of 16S rDNA was set as the internal control. The relative quantification analysis of *chrA*/*yieF* was performed using the $2^{-\Delta\Delta Ct}$ analysis, where ΔΔCt = [ΔCt (CT)–ΔCt (NT)] and ΔCt = [Ct (*chrA*/*yieF*) - Ct (16S rDNA)] (Arandi *et al.*, 2018). All analyses were performed in triplicate.

To evaluate the microbial biomass of the seven soil samples, the 16S rDNA gene was quantified using qPCR with gDNA, which was extracted by a Soil DNA Extraction Kit (MoBio), according to the manufacturer's directions. Different multiple dilutions of plasmid pMD-18T containing 16S rDNA fragment were used as a standard for calculating copy numbers (Liu *et al.*, 2019). The primers and processes were the same as described above.

Metatranscriptomic validation

The total microbial RNA of the mixed sample was sent to Majorbio, Inc., Shanghai, China. 5 μg of RNA in NT and CT was used as the requirement of Ribo-Zero Magnetic Kit (Epicenter, Madison, WI). First, rRNA was removed from the total RNA by Ribo-Zero rRNA Removal Kit (Plant Leaf) (Epicenter, Madison, WI, USA) and Ribo-Zero rRNA Removal Kit (Meta-Bacteria) (Epicenter). Poly(A) RNA was depleted using the Oligotex mRNA Kit (Qiagen, Hilden, Germany). Subsequently, the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA, USA) and cBot TruSeq PE Cluster Kit v3-cBot-HS (Illumina) were used for library construction and bridge PCR, respectively. Then, paired-end sequencing (2 × 150 bp) was performed on an Illumina HiSeq 4000 instrument (Illumina) using TruSeq SBS Kit v3-HS (Illumina). Raw metatranscriptomic data have been deposited in NCBI Sequence Read Archive (SRA) under the accession number SRP154829.

To obtain a clean read dataset, the following steps were performed: (i) Raw reads were trimmed by using SeqPrep (https://github.com/jstjohn/SeqPrep); (ii) reads with quality scores less than 20 and lengths below 50 bp were deleted using Sickle (https://github.com/najoshi/sickle); (iii) removed reads were aligned to a host, such as the human genome or animal faeces, using BWA (http://bio-bwa.sourceforge.net/); (iv) reads in the

metatranscriptomic libraries aligned to SILVA SSU (16S/18S) and SILVALSU (23S/28S) databases were filtered out using SortMeRNA (http://bioinfo.lifl.fr/RNA/sortmerna/).

Statistical analysis

Data were expressed as the mean ± SD. RT-PCR and qRT-PCR results were examined by Tukey's test. All statistical analyses were performed in SPSS 16.0 for Windows.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Characteristics of different types of heavy metal-contaminated soil samples.

Fig. S1. Summary of published papers in the past ten years in different research areas with the key words showed in the figure. Data were from Web of Science (http://apps.webofknowledge.com/).

Fig. S2. 16S rDNA copy number for different types of soil. Values with different letters mean significant differences at $P < 0.05$, as determined by Tukery's test.

Fig. S3. Quality and quantity of the total microbial RNA extracted from an uncontaminated flowerpot soil sample by the novel approach.

Fig. S4. Soil characterization of different types of soils.

Fig. S5. Total microbial RNA integrity of different contaminated soils extracted with the novel method was identified by 1.0% agarose gel electrophoresis.

