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Inactivation of antibiotic-resistant bacteria by chlorine dioxide in soil and shifts in community composition

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To study the efficacy of chlorine dioxide in the inactivation of antibiotic-resistant bacteria in soil, bacteria resistant to penicillin, amoxicillin or streptomycin were screened out from the soils around a hennery. The effects of dosage, contact time and pH value on the killing rates were investigated by batch experiments. The community composition before and after inactivation was analyzed by high-throughput genetic sequencing. The results showed that antibiotic-resistant bacteria are common and widespread in soil and the most resistant species is *Staphylococcus aureus*. More than 99% of antibiotic-resistant bacteria could be killed by chlorine dioxide at 5 mg L^{-1} within 30 min under neutral conditions. The killing log value declined slightly when the pH was changed from 4 to 9. The dominant genus was *Sphingomonas*, which was sensitive to chlorine dioxide and could be inactivated easily similar to *Arthrobacter* and *Massilia*. However, Micromonosporaceae and *Thaumarchaeota* were more resistant to chlorine dioxide than other species, and their relative abundance increased after disinfection.

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Introduction

Antibiotics have an essential role in controlling bacterial diseases in medical treatments and agriculture. They enter water and soil by the spreading of manure[1] or by direct excretion from livestock. Antibiotics then enter deeper soil layers by surface runoff, driftage or leaching[2] and finally accumulate in plants.[3] Many antibiotic-resistant bacteria (ARB) in soil have been found where livestock congregate. Manure application has significantly increased the diversity and abundance of antibiotic resistance genes (ARGs) in soil and also markedly shifted the bacterial composition that was significantly correlated with the ARG profiles.[4] Uncontrolled use of antibiotics has led to the enrichment of ARGs in manure[5] and affected environments, particularly soils.[6] In the U.S., 60% to 80% of all antibiotics were used in animal production, and more than 80% of them were excreted in manure,[7] which led to further pollution.

ARGs have been widely found in the soils of livestock areas. Zhu[5] found 149 kinds of ARGs in the soils around three large farms, which were about 192–28 000 times higher than those found in soils without the use of antibiotics. Although antibiotic resistance may decline after the relaxation of selection pressures, low yet detectable levels of resistance determinants are likely to persist for decades.[8] Also, the quantity of ARGs in the soil remains high even when the livestock has been removed from the site for two years.[9]

These ARB and ARGs from agricultural settings can be transferred to humans and become a critical health concern.[10] Completely antibiotic-resistant tuberculosis cases were reported in Iran in 2009 and India in 2011, independently. They were resistant to all the first- and second-line drugs.[11] An analysis of 264 soil isolates obtained from different natural habitats in and around Hyderabad has identified 5 isolates that are resistant to as many as 10 antibiotics.[12] Increased consumption of antibiotics may produce not only greater resistance at the individual patient level, but also greater resistance at community, country, and regional levels, which nevertheless can harm individual patients.[13]

It has been a hot topic to find out methods to slow or restrain spreading of drug resistance.[14] Most studies have reported the effect of various disinfection methods on ARB inactivation in water, and these methods include the use of ozone,[15] chlorine,[16] and UV. However, UV and ozone disinfections result in apoptosis, and the bacterial DNA is released into the environment; then, ARGs are mostly found as free DNA in the treated wastewater.[17] Very few studies are available about the effects of the disinfection process on the inactivation of ARB and ARGs in soils. Drug resistance to traditional disinfectants used in farms, such as phenol, formaldehyde,[18] sodium hypochlorite, calcium hypochlorite,[19] and quaternary ammonium compounds,[20] has been common due to the extensive application for many years. As the native soil bacterial species play a role in inhibiting the

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survival of ARB or dissemination of ARGs,[21] the restoration of the microbial ecosystem after disinfection is also very important not only for soil function but also for reducing antibiotic resistance. Calcined eggshell amendment mitigated mixed pollutant accumulation in bell pepper significantly and enhanced the dissipation of soil tetracycline, sulfadiazine, roxithromycin, and chloramphenicol; it also decreased the water-soluble fractions of antibiotics and the diversity of ARB/ARGs inside the vegetables and contributed to the significant restoration of microbial biodiversity and stability.[22]

Chlorine dioxide (ClO_2) has been widely used for the inactivation of microorganisms and the removal of organic compounds as a substitute of chlorine for henhouse and cowshed disinfection and for other purposes in animal husbandry.[23] After soaking in ClO_2, the hatchabilities of eggs and duck eggs increase by 2% and 4%, respectively, compared to that observed for fumigation with potassium permanganate and formaldehyde,[24] and the incidence of zoonosis decreases.[25] The C_t 99 values of ClO_2 and chlorine are similar for ARB, and the effects of ClO_2 disinfection are not affected by ammonia nitrogen.[14] Chlorine dioxide also has an excellent effect on the inactivation of intracellular ARGs,[26] which indicates that fewer ARGs would be released into the environment. Truchado[27] found that the use of low residual ClO_2 concentrations (approx. 0.25 mg L^{-1}) to treat irrigation water decreases the relative abundance of Pseudomonadaceae (2.28-fold) and Enterobacteriaceae (2.5-fold) when comparing treated *versus* untreated baby spinach samples. Members of these two bacterial families are responsible for food spoilage and foodborne illnesses.[28] There is still no report on ClO_2 killing antibiotic-resistant bacteria directly in soil.

In this paper, the effect of ClO_2 on the inactivation of ARB in soil near a henhouse was investigated, and the differences in the bacteria community before and after treatment were compared to provide a reference for the application of ClO_2 to reduce ARB and ARGs in the soil.

Materials and methods

Soil samples

Soil samples were collected at 4 places in the gardens and vegetable fields that were 2 km away from a hennery located at 119.241672E and 39.871822N in Qinhuangdao City in China. All the soil samples were collected 10–15 cm underground and then screened through a 35 mesh sieve (with a particle size of 425 μm) after drying.

In order to investigate the antibiotic resistance under severe contamination pressure, the natural soil obtained above was mixed equally and then contaminated by antibiotics in the laboratory. Then, an antibiotic solution (penicillin, amoxicillin or streptomycin) was added into a 50 mL centrifuge tube (filled with 25 mL sterile water and 1 g mixed soil sample) at the concentration of 1, 2.5, 5.0, 10, or 50 mg L^{-1}. The control group was prepared by the same procedure using sterilized water instead of antibiotics.

Chemicals

Chlorine dioxide was prepared by the reaction between H_2SO_4 and $NaClO_2$ (eqn (1)) and absorbed by pure water.

$$5NaClO_2 + 2H_2SO_4 = 4ClO_2 + 2Na_2SO_4 + 11NaCl + 2H_2O \quad (1)$$

Then, it was diluted and calibrated before use by sequential iodometry.[29] Sodium thiosulfate solution (0.05 mol L^{-1}), which was used to neutralize ClO_2 at the end of the treatment process, was prepared by dissolving 7.9 g sodium thiosulfate in 1 L pH 7 buffer solution. All the pH buffer solutions (pH 4–9) were prepared by using KH_2PO_4 and Na_2HPO_4. All the solutions were sterilized by autoclaving and stored at 4 °C before use.

Screening of antibiotic-resistant bacteria

Antibiotic-resistant bacteria were screened from natural soil and contaminated soil samples by using the plate streaking method. At first, 1 g of dried soil was soaked by 25 mL of sterile water. Then, it was centrifuged at 4000 rpm for 5 min. The bacterial supernatant prepared from different soils was then diluted 100-fold and spread onto 3 plates filled with nutrient agar medium. Three parallel plates were prepared for each sample. All the plates were incubated for 24 h at 37 °C in an HPS-400 biochemical incubator (Guowang Instruments Co., Ltd., Changzhou, China).

Antibiotic susceptibility was tested by the CLSI M100-S26 method using a drug resistance paper disc[30] (Hangzhou Microbial Agent Ltd., product no. S1001 for penicillin, no. S1079 for amoxicillin and no. S1031 for streptomycin). The results were reported as sensitive (S), intermediate (I) and resistant (R). All the resistant strains were identified by culture and biochemical tests[31] and the most resistant ARBs were further identified by 16SrDNA sequences.

Disinfection

The effects of chlorine dioxide (ClO_2) on ARB inactivation were investigated by the suspension quantitative germicidal test. An ARB suspension was quantified by spectrophotometry at a wavelength of 530 nm. Then, it was diluted to an absorbance of 0.350 before disinfection to keep the cell concentration at about 1×10^8 CFU mL^{-1}. The calibrated ClO_2 solution was added by 1.0 mL pipette to a 10 mL centrifuge tube filled with 4.0 mL of different diluted ARB suspensions. Sterile water was used as a positive control. The tubes were put into a shaking table at a constant temperature. After a certain time, 1 mL of 0.05 mol L^{-1} buffered sodium thiosulfate was added to neutralise the remaining ClO_2. Then, the plate counting method was used to count the residual bacterial after ten-fold serial dilution. Three parallel samples were made for each antibiotic-resistant strain, and the thalli concentration was reported (N). The killing log value (KL) was calculated by $\log(N_0/N_x)$. The killing rate (KR) was calculated as $KR = (1 - 10^{KL}) \times 100\%$.

Sequencing of 16S rRNA gene and bioinformatics analysis

Disinfection was performed by adding 25 mL 15 mg L^{-1} ClO_2 to 50 g of the mixed soil sample collected in the wild. Then, it was

centrifuged for 10 min at 4000 rpm. Total genomic DNA from the disinfected and raw soil samples was extracted using the CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels. The 16S rRNA genes of distinct regions (16SV4) were amplified using a specific primer (515F-806R) with the barcode.

All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). We mixed the same volume of 1× loading buffer (containing SYB green) with PCR products and operated electrophoresis on 2% agarose gel for detection. Samples with a bright main strip between 400 and 450 bp were chosen for further experiments. PCR products were mixed in equidensity ratios. Then, the mixture of PCR products was purified with a Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations and index codes were added. The library quality was assessed on a Qubit@2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq2500 platform and 250 bp paired-end reads were generated. Paired-end reads were merged using V1.2.7 FLASH.[32] Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags[33] according to QIIME.[34]

Sequence analysis was performed by using Uparse software v7.0.1001.[35] Sequences with ≥97% similarity were assigned to the same Operational Taxonomic Units (OTUs). For each representative sequence, the Green Gene Database[36] was used based on an RDP classifier (version 2.2)[37] algorithm to annotate the taxonomic information. The OTU abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences.

Results

Antibiotic susceptibility of ARB

ARB are found in every sample taken in different places irrespective of whether it is a natural or contaminated soil sample, and there is no significant difference. Bacteria that showed resistance to 3 kinds of antibiotics were found in all the samples, which indicated that resistance can be widely transferred. Moreover, 24 ARB strains were isolated from the soils (Table 1) altogether. Cross drug resistance was ubiquitous. All the penicillin-resistant strains were resistant to amoxicillin as amoxicillin is a kind of semi-synthetic penicillin.

The 7 strongest antibiotic resistant isolates were chosen for the disinfection experiment. P3, P4, and N6 isolates showed the strongest resistance to penicillin; A2 and N3 showed the strongest resistance to amoxicillin and S8 and N4 showed the strongest resistance to streptomycin. Their cross drug resistance is shown in Table 2. The results of the bacterial morphological examination indicated that all the colonies were round, smooth, non-transparent, faint yellow, neatly edged and wet. P3 and P4 were about 4–5 mm in diameter, and the others were about 3 mm in diameter. Based on 16S rDNA sequencing, all of the 7 isolates were found to be *Staphylococcus aureus* (SA).

Disinfection efficacy of chlorine dioxide on ARB

The effect of ClO_2 dosage on disinfection was investigated at concentrations of 2, 4, 6, 8 and 10 mg L^{-1} at 25 °C and pH 7.2 for 30 min. From the result in Fig. 1, it can be seen that the killing log values for all the antibiotic-resistant bacteria increase along with ClO_2 dosages. Streptomycin-resistant bacteria were more resistant to ClO_2 than penicillin- or amoxicillin-resistant bacteria. At 4 mg L^{-1} dosage of ClO_2, KL values were 2.6, 2.7, 2.8 and 2.5 for P3, P4, N6, and A2, while they were only 2.2, 2.1 and 2.0 for N3, S8, and N4, respectively. The KL values increased to 2.0 and 2.1 for streptomycin-resistant bacteria (S8, N4) at a dosage of 6 mg L^{-1}.

The kinetics of ClO_2 inactivation of antibiotic-resistant bacteria was investigated by fitting the killing number using the Chick–Watson law (eqn (2)). The C_t values and k values at 3 log killing rate are listed in Table 3. The C_t values were in the range of 23–28, which indicated that all the antibiotic-resistant bacteria could be easily killed by ClO_2.

$$\lg\frac{N_t}{N_0} = -kC_t \quad (2)$$

The experiments of the effect of contact time on disinfection were conducted at 5 mg L^{-1} ClO_2 dosage and pH of 7.2. The suspension quantitative germicidal test was terminated by sodium thiosulfate after contacting for 5, 10, 15, 20 and 30 min. From the results in Fig. 2, it can be seen that the KL values of all

Table 2 Cross-resistance of the strongest drug-resistant strains

Strains		P3	P4	N6	A2	N3	S8	N4
Antibiotic susceptibility	Penicillin	R	R	R	R	R	R	R
	Amoxicillin	R	I	R	R	R	S	I
	Streptomycin	I	R	R	I	I	R	R

Table 1 Sensitive, intermediate and resistant antibiotic-resistant strains screened out by the CLSI M100-S26 method

	Sensitive		Intermediate		Resistant	
	Natural soil	Contaminated soil	Natural soil	Contaminated soil	Natural soil	Contaminated soil
Penicillin	0	4	0	2	6	4
Amoxicillin	0	0	2	1	6	1
Streptomycin	2	5	3	2	3	2

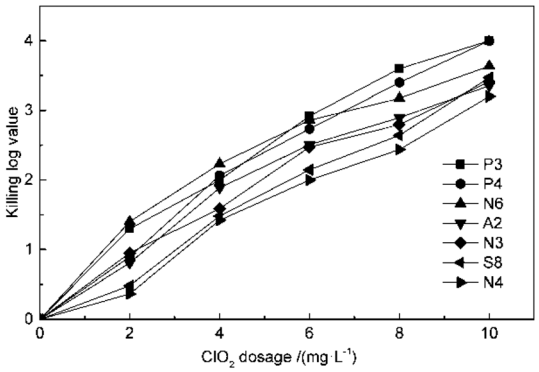

Fig. 1 Effect of ClO_2 dosage on killing antibiotic-resistant bacteria.

Table 3 Bacteriolytic kinetics of ClO_2 killing antibiotic-resistant bacteria at 3 log KL values

	P3	P4	N6	A2	N3	S8	N4
C_t (3 log)	23.65	25.83	23.33	27.06	26.05	24.36	23.71
k	0.127	0.116	0.129	0.111	0.115	0.123	0.127

the antibiotic-resistant bacteria increase for a contact time of 30 min. P3, P4, and A2 were killed by ClO_2 quickly. The KL values of P4 and A2 almost reached 1.0 after contacting for 5 min, while those of N3 and N4 were just beyond 1.0 after 10 min. The slopes of the KL value and contact time were all slightly down after 20 min, which may be because ClO_2 decreased with time.

The reactivation of ARB after disinfection was investigated at room temperature and under light conditions. The plates with 1 $\times$ 10^8 CFU mL^{-1} ARBs were treated by 5 mg L^{-1} ClO_2 for 30–50 min. The KL values of the 3 kinds of ARBs were all about 3 log. Then, they were calculated every 12 hours; the results are listed in Table 4. Twenty-four hours after ClO_2 disinfection, all the KL values of ARBs were maintained at 3 log. Isolate A2 showed reactivation and regrowth at 36 h, and its KL value decreased to 2.7. Then, P3 and P4 started reactivation and regrowth at 48 h; their KL values were also 2.7 and that of A2 continued to decrease to 2.5 at the same time.

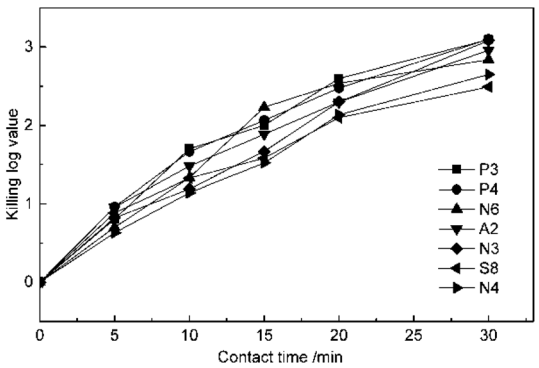

Fig. 2 Effect of contact time on antibiotic-resistant bacteria disinfection by ClO_2.

Table 4 Average killing log values of antibiotic-resistant bacteria 24 h after disinfection

	Strains						
Time/h	P3	P4	N6	A2	N3	S8	N4
12	3	3	3	3	3	3	3
24	3	3	3	3	3	3	3
36	3	3	3	2.7	3	3	3
48	2.7	2.7	3	2.5	3	3	3

The effect of pH value was studied at 4 mg L^{-1} ClO_2 and 15 min by changing the pH value to 4, 5, 6, 7, 8, and 9 using a phosphate buffer solution. The results in Fig. 3 indicate that the KL values slightly decrease in general in the pH range of 4–9. This may be because chlorite, a common by-product in ClO_2 disinfection, is a kind of oxoacid group and has a higher redox potential under low pH conditions. The KL values for P3, P4, and N6 were higher than the others at pH 4; then, those of P3, P4, and N3 decreased along with the increase in pH value, whereas the value for N6 first increased and then decreased. The KL values of S8 and N4 were not significantly affected until the pH value was raised to 9, which is due to the disproportionation occurring for ClO_2 beyond pH 9. However, the effect of pH on ClO_2 killing antibiotic-resistant bacteria was not significant in soil because the pH of common soils is between 4 and 9.

Analysis of bacterial community shift

Total 45754 and 66360 effective tags were collected in the extracts of disinfected and raw soil samples, respectively, from which 1608 and 1654 OTUs were clustered and annotated. From the relationship of OTUs between the two samples as the Venn graph demonstrates in Fig. 4 and the relative abundance of the species described in Fig. 5, it can be seen that there is no great difference between the species in the soil before and after ClO_2 disinfection. *Proteobacteria* dominated in both samples, followed by *Actinobacteria*. The relative abundances of *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Thaumarchaeota* significantly decreased after disinfection, while those of *Actinobacteria* and *Firmicutes* increased.

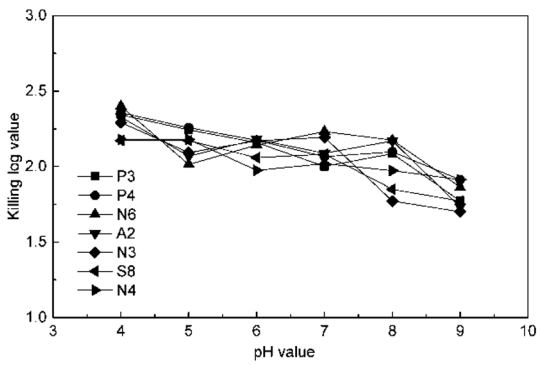

Fig. 3 Effect of pH on killing antibiotic-resistant bacteria.

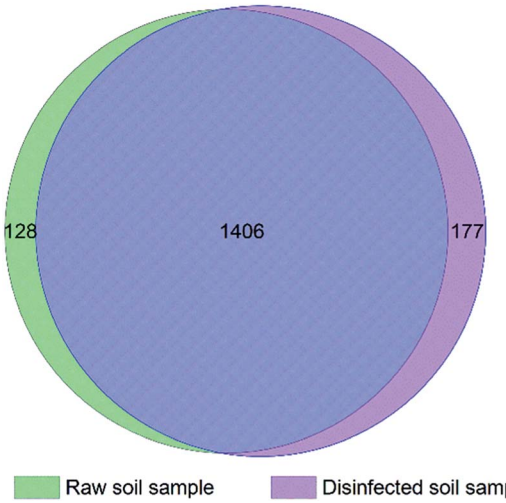

Fig. 4 Venn graph of clustered OTUs between the disinfected and the raw soil samples.

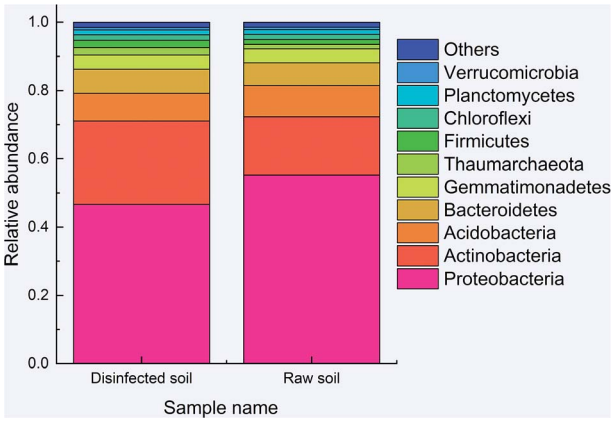

Fig. 5 The relative abundance of the species between disinfected and raw samples at the kingdom level.

The 50 most abundant OTUs in the heat map of Fig. 6 illustrate highly similar profiles of OTU frequencies for both samples, in which S1 represents a disinfected soil sample and S2 represents a raw sample. The dominant species in both samples were *Arthrobacter oxydans* and *Sphingomonas*, whose relative abundances decreased by 48% and 55%, respectively, after ClO_2 disinfection (from 1035 sequences to 636 and 346 + 990 + 461 + 320 to 188 + 502 + 285 + 185, respectively). The amounts of *Paucimonas*, *Massilia timonae*, and other species belonging to *Sphingomonadales* and *Piscinibacter* were also reduced. Furthermore, the relative abundance of Micromonosporaceae increased mostly (from 98 to 494) and that of *Blastococcus* also increased significantly (from 330 to 547). Other major species that increased after disinfection were *Steroidobacter* (from 225 to 396), *Microvirga* (from 241 to 390), and *Thaumarchaeota* (from 181 to 321).

Discussion

From the results mentioned above, we inferred that all the strains found in soil and having the strongest resistance were *Staphylococcus aureus* and not *Escherichia coli*, as reported before.[38–40] *Staphylococcus aureus* is a major pathogenic microorganism responsible for a series of infections. In particular, methicillin-resistant *Staphylococcus aureus* (MRSA) exists all around the world and has become a major pathogenic microorganism in hospitals and community.[41] Penicillin, amoxicillin, and streptomycin are 3 kinds of antibiotics that are commonly used in animal husbandry in China. Therefore, this proves that antibiotics are no longer as effective as before, and their long-term use has led to serious drug resistance.[42]

Chlorine dioxide can inactivate ARBs effectively and is an effective agent for soil disinfection. Although there have been many reports about ClO_2 inactivation of ARB and ARGs in water and wastewater in recent years, there is no such study about soil disinfection and no mention of multi-drug resistant MRSA. Judging by the 3 log C_t values, ClO_2 is a highly efficient

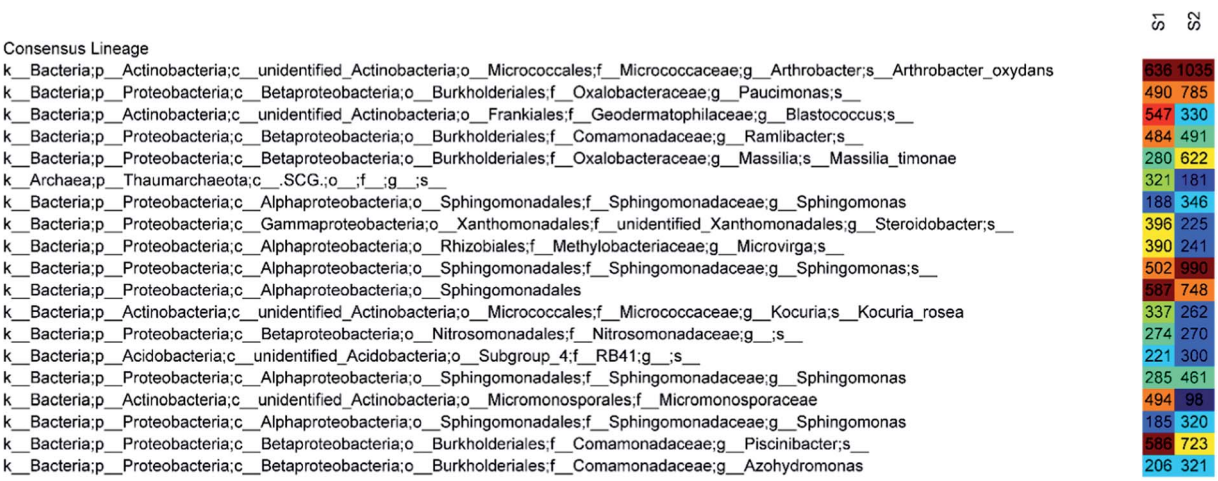

Fig. 6 Heat map of species annotation results of disinfected (S1) and raw (S2) soil samples. The numbers indicate the amount of sequences of the OTU in the sample and the colour represents the number of sequences of the OTU in the sample.

disinfectant not only for water but also for soil. Chlorine dioxide is more suitable for soil disinfection than NaClO.[43] It evaporates rapidly from an aqueous solution as a gas and then permeates the soil to contact and kill bacteria. However, HOCl and other liquid disinfectants have less permeability in the soil.[44] As the pH range of common soil is about 5–8, the effect of pH on the disinfection is negligible.

There is no great difference between the species in the soil before and after ClO_2 disinfection; this is consistent with the results of Truchado's study,[44] in which the abundance of *Proteobacteria* (the major genus), Pseudomonadaceae and Enterobacteriaceae decreased when comparing samples that were treated and untreated with ClO_2. It has also been reported that *Proteobacteria*, *Firmicutes*, and *Planctomycetes* are tolerant of chlorine in the secondary effluent of wastewater treatment plants.[45] Also, the relative abundances of *Pseudomonas* and *Sphingomonas* increase in the drinking water disinfection process after chlorination.[46] This is similar to the results observed for the use of ClO_2 in this study as phylum *Planctomycetes* is a kind of aquatic bacterium. *Sphingomonas* is sensitive to ClO_2 but not to chlorine.[47] This may be due to the good penetrating ability of chlorine dioxide, which can penetrate the fatty acid structure of the cell membrane of *Sphingomonas*.[48] Thus, the result suggests that ClO_2 is a better disinfectant for soil disinfection treatments than chlorine.

It should be noted that in this study, we only investigated the characteristics and changes in ARB in the soil during ClO_2 disinfection. The ability of ClO_2 to destroy ARGs still needs to be studied further.

Conclusion

In this study, we identified bacterial species that are resistant to penicillin, amoxicillin, and streptomycin in the soil around a hennery and the disinfection efficiency of ClO_2 on them. Bacteria resistant to penicillin, amoxicillin and streptomycin are common in natural soils. *Staphylococcus aureus* exhibited the strongest resistance. All the bacteria resistant to penicillin were also resistant to amoxicillin. ClO_2 could inactivate ARB in soils effectively, and the effect of pH value was not significant. Micromonosporaceae and the identified species in *Thaumarchaeota* were more resistant to ClO_2 than *Sphingomonas*, *Arthrobacter* and *Massilia*. At the phylum and class levels, no significant differences in the bacterial communities were observed between the untreated and ClO_2-treated soil samples. *Sphingomonas* was the dominant genus in the soil before and after treatment by ClO_2. Based on the results obtained, ClO_2 could be considered as a suitable disinfectant for ARB in soil.

Conflicts of interest

There are no conflicts to declare.

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