RESEARCH ARTICLE

Removal of sulfamethoxazole and trimethoprim from reclaimed water and the biodegradation mechanism

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HIGHLIGHTS

• The artificial composite soil treatment system could efficiently remove SMX and TMP by biodegradation mechanism.
• *Bacillus subtilis* from column reactors degraded SMX and TMP efficiently.
• *Bacillus subtilis* biodegrades TMP to NH_4^+, and then converts NH_4^+ to NO_3^- .

GRAPHIC ABSTRACT
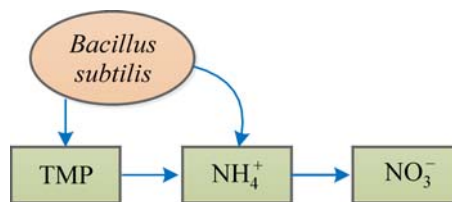
ABSTRACT

Sulfamethoxazole (SMX) and trimethoprim (TMP) are two critical sulfonamide antibiotics with enhanced persistency that are commonly found in wastewater treatment plants. Recently, more scholars have showed interests in how SMX and TMP antibiotics are biodegraded, which is seldom reported previously. Novel artificial composite soil treatment systems were designed to allow biodegradation to effectively remove adsorbed SMX and TMP from the surface of clay ceramsites. A synergy between sorption and biodegradation improves the removal of SMX and TMP. One highly efficient SMX and TMP degrading bacteria strain, *Bacillus subtilis*, was isolated from column reactors. In the removal process, this bacteria degrade SMX and TMP to NH_4^+, and then further convert NH_4^+ to NO_3^- in a continuous process. Microbial adaptation time was longer for SMX degradation than for TMP, and SMX was also able to be degraded in aerobic conditions. Importantly, the artificial composite soil treatment system is suitable for application in practical engineering.

1 Introduction

The occurrence and fate of sulfonamides in environment, including soils (Mojica and Aga, 2011; Pan et al., 2016), surface water (Pal et al., 2010), groundwater (Vulliet and Cren-Olivé, 2011; Chen et al., 2015; Sui et al., 2015;) and drinking water (Stackelberg et al., 2004; Westerhoff et al., 2005; Kleywegt et al., 2011) has drawn great attention of researches in recent years with the development of detecting techniques, especially LC/MS/MS (Hernández et al., 2007). Although it was reported that the concentra-

tion of sulfonamide antibiotics residue in the environment was low, usually at ng/L or μg/L level in surface water and wastewater due to the "pseudo-durability" of the antibiotics, sulfonamide antibiotics in the environment for a long time may result in the development of sulfonamide antibiotics resistant bacteria and antibiotics resistance genes in the long-term (Li and Zhang, 2010).

Municipal wastewater treatment plants (WWTPs) fail to eliminate organic micropollutants, and discharge of effluent containing these compounds is a major source of environmental pollution. Many organic micropollutants have the ability to resist the treatment in WWTPs. Consequently, the range of organic micropollutants in WWTP effluents would reach to the mg/L level (Loos et

al., 2013). Sulfamethoxazole (SMX) and trimethoprim (TMP) are frequently found in WWTP effluent because these drugs have enhanced persistency. The presence of TMP in WWTPs can generally be correlated to that of SMX because these two drugs are often co-administered. The highest reported absolute concentrations of TMP and SMX in the secondary effluent were 6.7 and 5 μg/L, respectively (Verlicchi et al., 2012). In Göbel et al. (2005) study, the sorption to activated sludge was shown to be low for sulfamethoxazole by activated sludge treatment and the effluent concentrations for sulfamethoxazole and clarithromycin were still 290 and 240 ng/L, respectively (Göbel et al., 2005). The removal efficiencies of SMX and TMP by WWTPs were –26% to 64% and –42% to 62%, respectively (Xu et al., 2007; Gulkowska et al., 2008). SMX appears strongly resistant to biodegradation in both surface water and WWTPs (Pérez et al., 2005).

The soil treatment systems are considered as the controlled application of wastewater into the soil surface to achieve a designed degree of treatment through natural physical, chemical, and biological processes within the soil- water matrix. In Vymazal et al.'s (2015) study, all outflow concentrations were lower than the limit of quantification of 0.5 ng/L in constructed wetland treatment system. Cristina et al.' (2015) study showed that a combination of horizontal subsurface flow, vertical subsurface flow and free water surface wetlands achieved high removal rates (above 80%) for PPCPs (Ávila and García, 2015). By the microbial molecular ecology techniques, Xu et al. (2011) constructed rapid infiltration system to reveal the removal mechanism of contamination and to improve wastewater treatment (Jiang et al., 2011). The natural soil purification process taking place during subsurface travel time led to the elimination of biodegradable micropollutants, and held the risk of micropollutants' migration from wastewater to waters (Weiss and Speth, 2003;Baumgarten et al., 2011). In this study, an artificial composite soil treatment system that can be applied in WWTPs to deal with SMX and TMP was provided.

An artificial composite soil treatment system was constructed, based on the concept of natural attenuation pathways like sorption and biodegradation, to process reclaimed water containing SMX and TMP as a model to overcome the incompletion removal of sulfonamides in WWTPs (Buser et al., 1998; Ziylan and Ince, 2011). Clay ceramsites with high sorption capability, volcanics with hydrophilic surfaces and coarse porosities, silty clay and coarse medium sands were chosen to be the filling material.

In a previous study, SMX is persistent in attempted biodegradations (Alexy et al., 2004; Benotti and Brownawell, 2009), whereas TMP can be biodegraded effectively by nitrifying activated sludge (Eichhorn et al., 2005; Batt et al., 2006). Batt et al. (2006) reported that the removal percentage of TMP decreased from 70% to 25% in batch reactors, when the activity of the nitrifying bacteria was inhibited (Batt et al., 2006). However, the detailed column experiment to study on the SMX and TMP biodegradation is rare and a detailed biodegradation pathway has not been determined. Meanwhile, to explain the SMX and TMP removal mechanisms and detail the biodegradation processes, NH_4^+, NO_2^- and NO_3^- concentration variations on reclaimed recharge processes were also investigated.

## 2　Experimental section

2.1　Chemicals and reagents

The chemicals used, suppliers, and purities are described in the supplementary. Physicochemical properties of the sulfonamide antimicrobials studied were reported in Liu et al. (2017a). The chemical composition of LB fluid nutrient medium was (g/L) peptone 10, yeast powder 5, NaCl 10 (agar plates added 1.2%–1.5% agar powder). The chemical composition of inorganic salt liquid medium was (g/L) NH_4Cl 0.2, NaCl 7.96, $MgCl_2 \cdot 6H_2O$ 0.8, $MgSO_4 \cdot 7H_2O$ 1.06, $CaCl_2 \cdot 2H_2O$ 0.1, KCl 0.22, $NaHCO_3$ 0.01, NaBr 0.026 and the added microelement liquid quantity was 0.5 mL/L. The yeast powder quantity is 5%. The chemical composition of microelement liquid component was (g/L) $Ca(NO_3)_2 \cdot 4H_2O$ 0.8, $CuSO_4 \cdot 5H_2O$ 0.08, $FeSO_4 \cdot 7H_2O$ 0.4, $ZnSO_4 \cdot 7H_2O$ 0.04, $MnSO_4 \cdot 4H_2O$ 0.04, $NaMO_4 \cdot 2H_2O$ 0.008, HBO_3 0.006. The pH of biodegradation experiment was 7.0–7.2. pH and DO were tested using potable water quality analyzer (HACH, USA).

2.2　Artificial composite soil columns set-up and operation

An overview of the artificial composite soil column' set-up is given in Fig. 1. Three polymethyl methacrylate artificial composite soil columns were set up to conduct the removal of SMX and TMP in the laboratory reported in Liu et al. (2017a). This experiment follows the experiment reported by Liu et al. (2017a), where 60-d artificial water with 20–30 μg/L SMX and TMP is used to study the adsorption capacity and the hydrogeological processes (Liu et al., 2017a). The influent concentration of the columns is about 20–30 μg/L. The detailed sampling process, the packing process of columns and the pretreatment process of columns are described in the supplementary. The experimental scheme of reclaimed-water recharge experiment is shown in Table 1. The laboratory temperature is about 20°C. The feed water was the reclaimed water from the Tsinghua University sewage treatment plant and the feed water in column 3 was added in 200 mg/L NaN_3 to inhibit the growth of microbes.

2.3　Biodegradation experiment

Soil samples (10 g) were removed from the medium of the
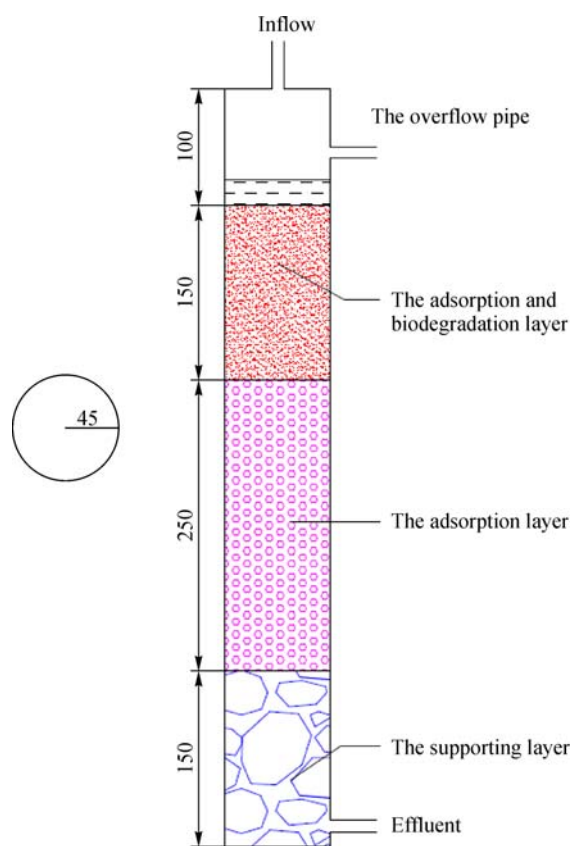
Fig. 1 Schematic diagram of the artificial composite soil column. The values are given in millimeter

biodegradation layers from column 1 after 100-d experiment and placed in 50 mL centrifuge tubes with sterilized water (20 mL). The centrifuge tubes were placed in a constant temperature shaker at 25°C and 140 r/min for 24h. Supernatant aliquots (100 μL) were taken and daubed to 5 mg/L SMX and TMP inorganic salt solid medium, respectively. All solid mediums were placed in a constant temperature incubator at 30°C and observed for microorganism growth. When colony morphology appeared, microbes were given in liquid inorganic salt media added 5% yeast powder. A 1-mL sample was taken daily to test the concentrations of SMX and TMP. Microbial strains were separated and purified using the spread-plate method

(Taylor and Geldreich, 1983). Biodegradation experiments were carried out in duplicate.

2.4 Analytical methods

2.4.1 UPLC/MS/MS analytical method

Samples were analyzed by liquid chromatography with tandem mass spectrometry (LC/MS/MS) using a LCQ Advantage ion trap mass spectrometer (ITMS) equipped with an electrospray ionization source (ESI) operated in positive ion mode (Waters, USA). The gradient elution program and antibiotics working parameter in mass spectrum is reported in Liu et al. (2017b).

2.4.2 Ammonia, Nitrite and nitrate analytical method

Ammonia, Nitrite and nitrate analyses were carried out according to Standard Methods (Association, 1960). Ammonia was measured by Nesslerization reagent spectrophotometry. Nitrate and nitrite were detected by a standard colorimetric method using a spectrophotometer (DR/6000 Spectrophotometer, HACH Company, Loveland, Colorado).

2.4.3 DNA extraction from isolated bacteria and identification of isolates

Resistant bacteria were picked from agar plates based on color features and unique colony morphology. The colony was cultured in LB liquid medium at 30°C, with 140 r/min for 12 h. Cells were obtained by centrifugation at 12000 r/min for 5 min, and 400 μL of lysis solution containing Tris (40 mM), EDTA (1 Mm), NaAc (20 mM) and 1% SDS was added to the mixture. Then 300 μL 5 mol/L NaCl was added and mixed by gentle vibration. After the mixture was centrifuged at 12,000 r/min for 20 min, the water phase was extracted first with a mixture of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) and then once with chloroform. The DNA was first precipitated with ethanol, and then washed with 70% ethanol. After dried and dissolved in 30 μL Tris-EDTA buffer (pH 8.0), it was stored at –20°C. The identification of resistant isolates was

Table 1 The experimental scheme of reclaimed water recharge experiment

Column codes	Column 1	Column 2	Column 3
The adsorption and biodegradation layer	Volcanics: silty clay: coarse medium sands v:v:v = 5:5:1	silty clay: coarse medium sands v:v = 10:1	silty clay: coarse medium sands v:v = 10:1 (200 mg/L NaN$_3$)
The adsorption layer	clay ceramsites	clay ceramsites	clay ceramsites
The supporting of layer	cobblestones	cobblestones	cobblestones
0.01 mol/L CaCl$_2$ deionized solutions	10 d	10 d	10 d
20–30 μg/L SMX and TMP reclaimed water solutions	60 d	60 d	60 d

referred to Xu et al. (2011).

3   Results and discussion

3.1   The TMP removal result

The variations of TMP concentration in the influent and effluent of different column systems throughout the removal process are depicted in Fig. 2(a).

The broad trends of the effluent concentrations in non-sterile columns were both downward and dropped drown more sharply in the later of the experiment. However, there is an uptrend in the effluent concentrations of column 3 in the middle-late stage. It showed that biodegradation is little in column 3, related to the influent fed with 200 mg/L NaN_3. Therefore, the main removal mechanism of columns 1 and 2 was biodegradation. Biodegradation was observed from the beginning of recharge experiment in column 1, but was hindered in column 3 by addition of 200 mg/L NaN_3. The results might also be attributed to the 60-d experiment exploring the hydrogeochemical characteristics of these three columns before the biodegradation experiments (Liu et al., 2017a). The removal rate in column 3 during this period was mainly attributed to sorption and the detailed sorption description is referred to Liu et al. (2017a). On the whole, the removal rate of column 1 is higher than column 2 and the removal rate of column 2 almost equals the column 3′ in the first 15 d. It might be related to the biodegradation layer material. Volcanics, silty clay and coarse medium sands were added in the biodegradation layer of column 1 while silty clay and coarse medium sands were added in column 2 and column 3. TMP effluent concentration is lower than that of SMX in the early-stage, which is consistent with the adsorption

experiment results reported by Liu et al. (2017a). After 45 d, the removal obviously increases in both non-sterile columns, which might be explained by adaption of the microbial community. The TMP removal efficiency of columns 1 and 2 finally reach more than 90%. Hijosa-Valsero et al. (2011) showed TMP removal efficiencies of 65%–99% by constructed wetlands (Hijosa-Valsero et al., 2011). It seems that the artificial composite soil treatment system is another efficient choice for TMP removal, compared with constructed wetlands. TMP is more readily biodegraded than SMX in the reclaimed water recharge process which might be related to the chemical properties of these two materials.

TMP can be biodegraded effectively by nitrifying activated sludge (Eichhorn et al., 2005; Batt et al., 2006). In this study, TMP removal in combination with NH_4^+, NO_2^- and NO_3^- removal was analyzed. The variations in NH_4^+, NO_2^- and NO_3^- effluent concentrations during the reclamation processes are described in Fig. 3. Nitrification in column 1 is more obvious than that of the other two artificial composite soil columns at the beginning of reclaimed water recharge, which corresponds with the TMP removal result. It was inferred that the TMP might be removed by the nitrifying bacteria like other nitrogenous organic compounds. Therefore, after 60-d operation, three columns were ceased for 20 d. Then three columns were recharged with reclaimed water again. High NH_4^+ concentrations were detected in columns 1 and 2. These high NH_4^+ concentrations (Table 2) lasted for two days and then returned to normal. The feed water was the reclaimed water from the Tsinghua University sewage treatment plant and had a deep treatment, so the total nitrogen was almost equal to the sum of NH_4^+, NO_2^- and NO_3^- nitrogen. Two samples were randomly chosen and the total nitrogen, NH_4^+, NO_2^- and NO_3^- concentrations were tested,
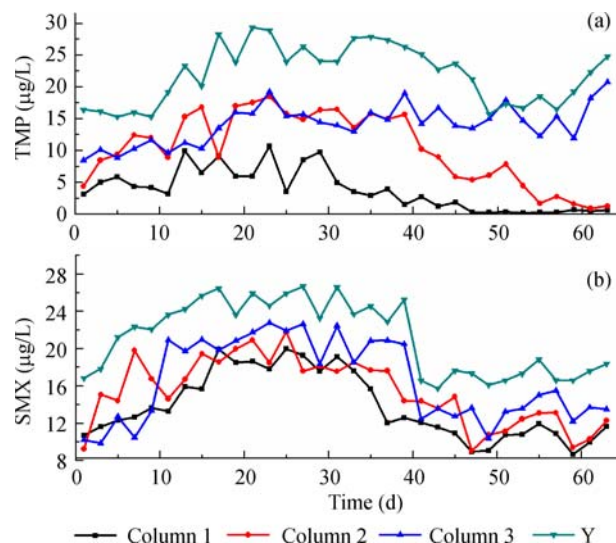
Fig. 2   The variation of SMX and TMP influent and effluent concentrations. Y represents the influent of columns 1, 2 and 3. Columns 1, 2 and 3 represent the effluent of columns 1, 2, and 3, respectively
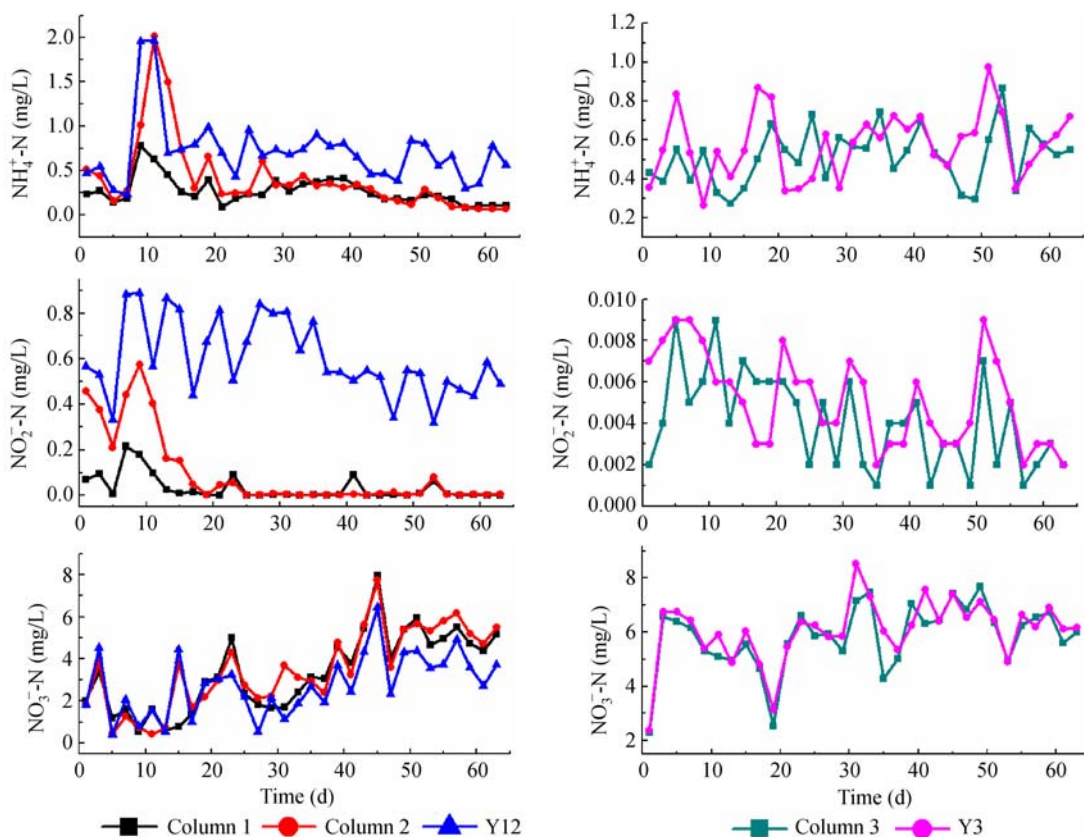
Fig. 3 The variation of NH_4^+, NO_2^- and NO_3^- influent and effluent concentrations. Y represents the influent of columns 1, 2 and 3. Columns 1, 2 and 3 represent the effluent of columns 1, 2, and 3, respectively

respectively (Table S1 in Supplementary Material). Therefore, the high NH_4^+ concentrations seen in column 1 and 2 could not be from the influent nitrogenous organic compounds, and the NH_4^+-N must come from TMP and SMX adsorbed in columns. The NH_4^+ concentrations dropped substantially after two-day monitoring, which also verified that the extra NH_4^+ was derived from adsorbed TMP and SMX. The NO_3^- effluent concentrations are also much higher than normal during the period of high NH_4^+ effluent concentrations (Table 2). It is speculated that TMP and SMX biodegradation occurs via a nitrification process, where nitrifying bacteria degraded SMX and TMP to NH_4^+ and further convert NH_4^+ to NO_3^-. Therefore, the SMX and TMP biodegradation microorganisms were cultivated to confirm that the extracted strains could conduct the nitrification process. Finally, details of the biodegradation process were further provided.

3.2 The SMX removal result

SMX monitoring results are shown in Fig. 2(b). The influent and effluent SMX concentration variation curves for the 60-d reclaimed water recharge experiment show that SMX was removed in column 1 (the average removal rate, 35.38%) slightly better than columns 2 (the average

Table 2 The high NH_4^+-N and NO_3^--N effluent concentration result

Effluent concentration	Column 1	Column 3	Column 5
NH_4^+-N (83 d)	15.62	11.52	1.84
NH_4^+-N (85 d)	9.16	6.87	1.68
NH_4^+-N (87 d)	1.13	1.36	0.92
NO_3^--N (83 d)	10.62	10.52	2.54
NO_3^--N (85 d)	8.16	8.87	3.68
NO_3^--N (87 d)	4.01	3.98	3.07

removal rate, 27.71%) and 3 (the average removal rate, 22.67%). Although column 1 has more volcanics in the biodegradation layers, the biodegradation was poor, compared with the TMP removal. The poor SMX removal effect of this artificial composite soil column might be related to biological degradability, unlike TMP removal. Heberer et al. (2008) showed that SMX was eliminated more rapidly under anoxic infiltration conditions (Heberer et al., 2008) and Grönheid et al. (2005) have supported this result (Grönheid et al., 2005). Therefore, O_2 content might affect SMX removal. Dissolved oxygen (DO) concentration in the influent and effluent are shown in Fig. 4. The DO concentrations in effluents are obviously higher than those in the influents, showing that all the artificial

composite soil columns are in an aerobic condition. SMX biodegradation could require anoxic condition.
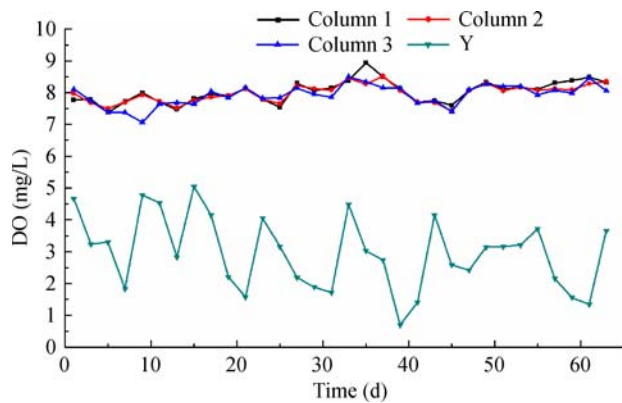
Fig. 4 The variation of DO influent and effluent concentrations. Y represents the influent of columns 1 and 2. Columns 1 and 2 represent the effluent of columns 1 and 2, respectively

Baumgarten et al. (2011) reported that about 60% of SMX in surface water was removed within 14 d of column passage under aerobic conditions, while no removal occurred under anoxic conditions (Baumgarten et al., 2011). It is likely that SMX degradation will require a long time under aerobic conditions. Baumgarten et al.' (2011) study showed that the adaptation time was very long and was not complete after 2 years of operation (Baumgarten et al., 2011). The three columns were rested for 20 d after 60 d experiment. To extend the microbial adaptation, columns 1 and 2 were operated again and the SMX removal results after this 20-d period are depicted in Fig. 5. Obvious biodegradation occurred in the columns during the extended experiment. The adaptation time of 91 d was shorter than that seen by Baumgarten et al. (2011), because the influent in this study was reclaimed water, while surface water was used by Baumgarten et al. (2011) and the soil in Baumgarten's columns was placed in a cooling chamber. It is concluded that the microbial adaptation time for SMX degradation is longer and SMX could be degraded in aerobic conditions.

On the whole, artificial composite soil treatment system could efficiently remove SMX and TMP. Moreover, the main removal mechanism in the later phase is the biodegradation. The technology of artificial composite soil treatment system is simple and the material is low-cost so that the artificial composite soil treatment system is suitable to apply to practical engineering.

3.3 Extraction and identification of strains

The SMX and TMP degrading microbes were isolated from column 1 after 100 d to further prove the detailed biodegradation process. Microbial colonies grew after five days of the cultivation and SMX/TMP could be degraded
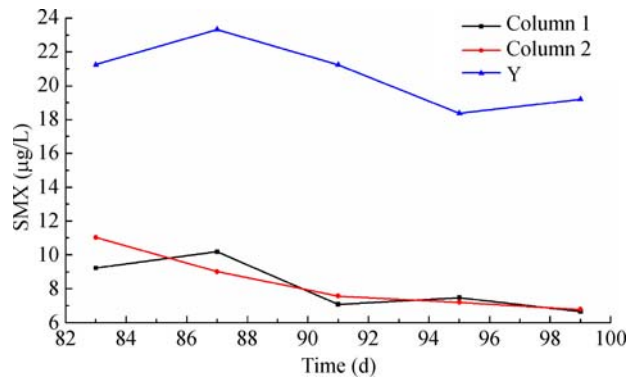
Fig. 5 The variation of SMX influent and effluent concentrations in additional experiment. Y represents the influent of columns 1 and 2. Columns 1 and 2 represent the effluent of columns 1 and 2, respectively

by 70% on the tenth day. One highly efficient degradation bacteria strain was isolated with one colony type formed in elevation and surface on agar (Fig. S1, see in Supplementary Material). The strain identification result was listed in Table 3. The bacteria that could efficiently degrade SMX and TMP are *Bacillus species* and it could be *Bacillus subtilis* with the 99% identity. *Bacillus species* are able to remove nitrogen and phosphorous as well as organic matter efficiently (Choi et al., 2002). However, bacillus strains that degrade antibiotics are very rare. Bacillus strains are known to be involved in heterotrophic nitrification. Heterotrophic Bacillus strains were able to occur in simultaneously aerobic nitrification/denitrification conditions (Choi et al., 2002; Kim et al., 2005). This further supports the speculation that TMP biodegradation occurs by a nitrification process, where the nitrifying bacteria degraded TMP to NH_4^+, which is further converted to NO_3^-.

Table 3 The result of identification for SMX and TMP resistant bacteria in artificial composite soil treatment columns

Closest species	Accession numbers	Identity (%)
Bacillus subtilis	KX953869.1	99

3.4 NH_4^+, NO_2^- and NO_3^- concentrations over time

Ammonia effluent concentrations from column 1 were low in the early phase of the experiment, which showed that nitrification had occurred (Fig. 3). It might be related to the process of artificial water recharge to study the adsorption removal and hydrogeological processes so that might result in nitrifying bacteria adaptation, referred to Liu et al. (2017a). There might also been nitrification in column 2, albeit weaker than in column 1. Nitrification in column 2 was obvious after 15 d, resulting in TMP biodegradation in column 2. It might be related to the components in the biodegradation layers of the columns. Column 1 contains

more volcanics in the biodegradation layer than columns 2 and 3. Volcanics with hydrophilic surfaces and coarse porosity contributed to microbial adhesion. The effluent ammonia concentrations from column 3 were almost the same as the influent concentrations because 200 mg/L NaN_3 was added to the influent of column 3, and nitrification was inhibited. The influent and effluent nitrite concentrations were also about the same and almost 0, because of the added NaN_3. The nitrite influent concentration ranges of column 1 and 2 are large, but the nitrite influent concentrations are all less than 1 mg/L. Nitrite effluent concentration is almost 0 by day 17 from columns 1 and 2. Nitrite effluent concentration variation might be related with nitrification.

Nitrate effluent concentrations from column 3 almost equal the influent concentrations (Fig. 3), and these are slightly higher than those of the other two columns. The reclaimed water is from the Tsinghua University sewage treatment plant and has undergone an advanced treatment process to remove almost all organic nitrogen sources. It was ruled out that organic nitrogen as a source that is converted to nitrate. The NaN_3 added to the influent of column 3 might result in the increase in the concentrations of nitrate nitrogen. The low influent ammonia concentrations make any trend of nitrate effluent concentrations not obvious. Nitrification is not obvious in the early phase of the experiment and there is almost no change in the nitrate effluent concentrations. The high nitrate nitrogen concentrations in the late stage of the experiment are caused by nitrification.

4 Conclusions

The artificial composite soil treatment system could efficiently remove SMX and TMP by biodegradation mechanism, and possess good application prospect in actual engineering. TMP is accessible to microbial degradation in the artificial composite soil treatment system. The strain identified that efficiently degraded SMX and TMP was *Bacillus subtilis*. The concentrations of NH_4^+, NO_2^- and NO_3^- in the columns show obvious nitrification, corresponding to TMP biodegradation. *Bacillus subtilis* first degraded TMP to NH_4^+, and then convert NH_4^+ to NO_3^- by nitrification, which explains the previously reported removal phenomenon that TMP was biodegraded effectively by nitrifying activated sludge. Microbial adaptation time for SMX degradation is longer than that for TMP, and SMX is also able to be degraded in aerobic conditions.

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