Effects of Sewage Effluent Recharge on The Microbial Community in A Soil Column Model

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Abstract

Soil microbial community shifts caused by feeding with secondary treated sewage effluent were analyzed to clarify the effect of artificial groundwater recharge on soil bacteria. A soil column system was used as the experimental model of artificial groundwater recharge, and secondary treated sewage effluents (nondisinfected, chlorinated, or ultraviolet disinfected) were fed into the columns. Soil microbial community shifts were analyzed by polymerase chain reaction – denaturing gradient gel electrophoresis (PCR–DGGE), and degradation of organic matter by soil microbes was monitored by measuring the dissolved organic carbon (DOC) concentration in the column outflow. Analysis of the PCR–DGGE profiles showed that the soil microbial community fed with disinfected effluents changed transiently, but the community shifted closer to the one of day 0 after day 30 on. The DOC removal efficiency once deteriorated by day 28, most probably due to the limitation of sorption. However, it started recovering with the recovery of the initial microbial community after day 41. This indicated that from day 41 onward DOC removal by biological degradation compensate for the limitation of that by sorption to soil particles. Our findings suggested that a soil microbial community that is fed with sewage effluent can retain its ability to remove organic matter. Besides, the disinfection method used on the effluent did not make much difference in the effect on the soil microbial community shift and the DOC removal efficiency.

Keywords: sewage effluent, soil column, soil microbial community, disinfection, artificial groundwater recharge

INTRODUCTION

Urban water supplies tend to be limited by the availability of water resources outside urban areas, such as water reservoirs. Artificial recharge of groundwater, a method that is well known for its purification capability, is a promising method for establishing sustainable water resources in urban areas9. Sewage effluent, which is abundant within urban areas, is one possible water source. However groundwater recharge with sewage effluent may cause groundwater and soil pollution as treated effluent contains organic substances, chemicals, viruses, and fecal bacteria2–6. In addition, disinfection of sewage effluent may increase toxic substances, such as chlorinated by-products, in spite of reducing the number of pathogens9. For example, a bisphenol A solution chlorinated with 1.46 mg l–1 sodium hypochlorite exhibited a 24-fold increase in estrogenic binding affinity compared to the nonchlorinated solution9. Likewise, many other phenolic compounds that exist in sewage effluent, such as pharmaceuticals, are reactive with

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hypochlorite.

To sustain the purification ability of soil during groundwater recharge with treated sewage, it is necessary to retain the biodegradation capacity of the soil bacteria because of the limited filtration or sorption capacity of soil particles. From the perspective of water reuse, the transports of pathogens, viruses, and bacterial genes in groundwater have been well examined\cite{5,9-11}, besides the elimination of pollutants\cite{12-16}. Harvey et al.\cite{17} ran the field research about the effect of secondary sewage effluent on the population of free-living and particle-bound bacteria and distribution within aquifer (soil and groundwater). Mancino et al.\cite{18} demonstrated change in total population of soil aerobic bacteria. Cho et al.\cite{19} showed the effect of livestock wastewater on the bacterial diversity in groundwater. Oved et al.\cite{20} investigated the soil bacterial community receiving secondary sewage effluent, targeting especially on composition and function of ammonia-oxidizing bacteria. However, little is known how indigenous soil bacteria react with the injection of sewage effluent. Moreover, the different disinfection method for the sewage effluent can affect on its microbial community differently\cite{21}. The effect of differently-disinfected effluent on soil microbial community is yet unknown either. Therefore, investigations of the effects of groundwater recharge with effluent treated by different disinfection methods on soil microbial communities are needed so that we can better understand the sustainability of soils for this application.

We used the PCR–DGGE method\cite{22,23} to analyze the soil microbial community shifts that occurred in soil columns fed with secondary treated effluent. In addition to analyzing the effects of disinfection, the effects of disinfection method used on sewage effluent—chlorination and ultraviolet (UV)–disinfection—on soil microbial communities were compared.

**MATERIALS AND METHODS**

**Column experiments** We used the PCR–DGGE method to analyze the soil microbial community shifts that occurred in soil columns fed with secondary treated effluent. DOC concentrations of outflow solutions were measured to monitor the degradation of organic matter by soil microbes. In experiment 1, we analyzed the effects of three kinds of inflow solution (secondary treated sewage effluent before and after chlorination, and tap water) on soil microbial communities. Sequences of bacterial 16S ribosomal RNA (rRNA) gene obtained from DGGE bands were analyzed, and the behaviors of bacteria in soil columns receiving each inflow solution were interpreted. In experiment 2, three kinds of inflow solution (chlorinated, UV–disinfected, and nondisinfected secondary treated sewage effluent) were used to compare the effects of disinfection method on soil microbial communities. In both experiments, soil microbial community shifts analyzed by PCR–DGGE were summarized with multidimensional scaling (MDS) correlation.

**Soil materials** For experiment 1, Andosol-like soil, which is widespread in eastern Japan, was collected to a depth of 10 cm from the surface at the experimental farm of the University of Tokyo. For experiment 2, Andosol-like soil was collected from a depth of 50 cm to 90 cm from the surface of a park in Nerima Ward, Tokyo. As soil and sewage effluent were different in each experiment, we did not compare these results. In both experiments, the soil was sieved to remove particles larger than 2–mm in diameter, and each sample was mixed uniformly.

**Inflow solutions** In experiment 1 we analyzed the effects of three kinds of inflow solution—secondary treated sewage effluent before and after chlorination, and tap water—on soil microbial communities. The two kinds of secondary treated effluent were obtained once every 2 weeks from a wastewater treatment plant in Japan. This plant receives mainly domestic sewage, and employs a conventional activated sludge process. Tap water, a control without treated sewage components, was taken to a beaker in the lab and left for 24 h to remove the free chlorine before being fed into the soil columns. In experiment 2, secondary treated effluent before disinfection was obtained weekly from the same wastewater treatment plant. Sewage effluent was divided into three parts:
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one part was left untreated, one was treated by chlorination, and one by UV–disinfection. Chlorination was carried out by adding sodium hypochlorite (Kishida Chemical Co. Ltd., Osaka, Japan) to nondisinfected sewage effluent to a final concentration of 3 mg l⁻¹, and the chlorinated effluent was left for 24 h to remove the free chlorine. UV–disinfection was carried out by irradiating 500 ml of nondisinfected sewage effluent with 38.4 mWs cm⁻² UV under dark conditions. In both experiments, the inflow solutions were stored at 4°C up to two weeks.

**Soil columns** Acrylic tubes with a length of 150 mm and an internal diameter of 25 mm were used as soil columns (Figure 1). Ten soil columns were prepared for each inflow solution. Before the columns were filled, 30 g of soil and 7 ml (or 5 ml in experiment 2) of inflow solution were mixed to a uniform consistency to obtain reproducible conditions and to avoid irregularity in the stream of water. The columns were filled with the prepared soil and sealed with silicon stoppers at both ends.

Each inflow solution was pumped into a container to ensure uniform solution quality in all columns and then allowed to percolate though the 10 columns by gravity. The inflow solutions were fed into soil columns for 4 weeks in experiment 1 and for 50 days in experiment 2. In order to keep aerobic condition in the soil columns, inflow solutions were kept aerobic by being mixed in the head tank before infiltration. Moreover, the retention time of inflow solutions in the soil columns were within 1.5 days, and, soil was not saturated with water. The whole column system was operated under dark conditions in experiment 2 to avoid photoreactivation of bacteria in the UV–disinfected sewage.

The outflows from the columns were collected in conical centrifuge tubes (BD Biosciences, NJ, USA), and the infiltration rates were calculated on the basis of the effluent volume and the time required to pass through the column. At each sampling time, two columns were sacrificed for soil sampling to examine the representative microbial community change in each treatment. Soil sampling was performed on days 1, 7, 14, and 28 in experiment 1 and on days 1, 8, 20, 30, 40, and 50 in experiment 2.

**DOC** To examine the efficiency of removal of organic matter from sewage effluent by permeation through the columns, DOC concentrations of the inflow and outflow solutions were measured. Before measurement, each solution was filtrated through a membrane filter with a 0.2-μm pore size. The filtrates were acidified with 40 μl of 2N HCl to about 5 ml of them and applied to a TOC–5000 total organic carbon analyzer (Shimadzu Co., Kyoto, Japan).

**DNA extraction and purification from soil** In both experiment 1 and 2, total nucleic acids were extracted from 0.5 g of soil with an ISOIL for Beads Beating Kit (Nippon Gene Co. Ltd., Tokyo, Japan). A FastPrep bead-beating machine (BIO 101, Holbrook, NY, USA) was used for mechanical disintegration of soil bacteria. The final nucleic acid pellet was dissolved in 50 μl of sterile Tris–EDTA buffer and used as a template for subsequent PCRs.

**PCR-DGGE** Oligonucleotides of the variable V3 region of bacterial 16S rRNA gene were amplified with two bacterial universal primers. The nucleotide sequences of primers were as follows: forward primer 357fGC, 5’–CGCCCGCCGCCGGCCGCGGC CGGGGCGGGGACGGGGGGGCTACGGAG GGCAGCAC–3’; reverse primer 518r, 5’–ATT ACCGCGGCTGCTGG–3’. Primer 357fGC had a 40–base guanosine-cytosine clamp at the 5’ end²⁰,²¹.

The PCR mixture contained 0.2 μM of each
primer, 200 μM of each deoxynucleoside triphosphate, 5 μl of 10 PCR buffer with 1.5 mM of MgCl₂, 1.25 U of Taq DNA polymerase (AmpliTaq Gold; Applied Biosystems, CA, USA), 400 ng μl⁻¹ of bovine serum albumin, and sterile Milli-Q water to a final volume of 50 μl. PCR was initiated for 10 min at 95°C; this was followed by 35 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C, and a final cycle of 10 min at 72°C. Amplification products were analyzed by electrophoresis in a 1% (w/v) agarose gel and ethidium bromide staining.

DGGE was performed with the D-Gene System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Samples (20 μl) were loaded onto 8% (w/v) polyacrylamide gels with a denaturing gradient ranging from 35% to 65%, where 100% denaturant contains 7 M urea and 40% formamide. The electrophoretic charge was applied in the same direction as the denaturants. All gels were run in 1× TAE buffer for 8 h at 60°C and 120 V. After electrophoresis, gels were stained for 15 min with VistraGreen nucleic acid gel stain (3 μl of a 10⁴-fold concentrated solution in 30 ml; GE Healthcare UK Ltd., Buckinghamshire, England) and immediately photographed by using a FluorImager595 (Molecular Dynamics, Sunnyvale, CA, USA) and Image Analysis Software (ImageQuaNT, Molecular Dynamics).

DGGE bands were carefully excised with a cutter and transferred to a 0.5-mℓ tube; 50 μl of sterile Milli-Q water was added, and the tube was frozen at 20°C overnight. After thawing to room temperature, the DNA solution was used as template for reamplification of the DNA fragment with the same primer set described above, and the amplicon was analyzed by doing DGGE. This process was repeated several times until a single band at the same position was obtained; gels with denaturing gradients ranging from 40% to 55% were used for separating each band more precisely.

Statistical analysis Changes in a soil microbial community were assessed by using dissimilarity data in the DGGE band patterns in conjunction with MDS analysis. MDS constructs a spatial configuration map of data points. On a MDS map each band pattern is represented as one plot, and relative changes in a community structure can be visualized and interpreted as the distance among plots. Distance between data points reflects the relationship between individual variables in the underlying data sets. In this study, the distance among plots was defined on the basis of dissimilarity index of DGGE band patterns between every two lanes.

The DGGE bands detected in each gel lane (a, b, ...) were numbered serially (Bᵃᵏ, Bᵇᵏ, ...) with the commonly defined band position (k). Bands not shown in the entire lane were also numbered in the same manner. The 21 major DGGE bands in each sample were used for MDS analysis in experiment 1, and 43 major bands in experiment 2. Fluorescence intensity of the band Bᵃᵏ was defined as Iᵃᵏ. Proportion of the band existence in a lane (Pᵃᵏ, Pᵇᵏ, ...) was calculated as follows:

\[
Pᵃᵏ = \frac{Iᵃᵏ}{\sum_k Iᵃᵏ} × 100 \quad (i)
\]

Dissimilarity index (D) of band patterns between lane a and b was defined as follows:

\[
D(a, b) = \frac{1}{2} \sum_k |Pᵃᵏ − Pᵇᵏ| \quad (ii)
\]

On the basis of dissimilarity index, DGGE band patterns in each lane were represented on a MDS map. Calculation was performed with SPSS software version 11.0J (SPSS, Chicago, IL, USA).

Sequencing DNA fragments obtained from each DGGE band were purified with a Montage PCRμ96 Purification Kit (Millipore Co., Billerica, MA, USA). Sequencing reactions were performed with ABI BigDye (version 3.1; Applied Biosystems) and the V3 region-specific primers 357f (5’-CCTACGGGAGGCAGCAG-3’) and 518r22, 23), with slight modifications to the manufacturer’s instructions. The sequencing products were purified with the PCRμ96 Purification Kit and analyzed with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Phylogenetic analysis Homology search of nucleotide sequences were performed by using the BLAST network services of the
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DDBJ database (DNA Data Bank of Japan; available via http://www.ddbj.nig.ac.jp/Welcome-j.html). The sequences were aligned by using the CLUSTAL X program version 1.83 (http://bips.u-strasbg.fr/fr/Documentation/CLUSTALX/), and phylogenetic trees were constructed by the neighbor-joining method. Trees were reconstructed and viewed with the NJPLOT program pack (http://pbil.univ-lyon1.fr/software/njplo.html).

RESULTS

Infiltration rates Infiltration rate is dependent on the hydraulic conductivity of the soil layer. Figure 2 shows the infiltration rates of all columns in experiment 1. The profiles of the changes were nearly the same in all columns. After 10 days of infiltration, the average rates of three conditions became steady at about 1 – 2.5 mm h⁻¹. The infiltration rate of tap water was higher than that of sewage effluents. Likewise, the infiltration rate became unchanged at about 1.5 mm h⁻¹ in experiment 2 (data not shown).

Efficiency of removal of organic matter by soil In experiment 1, the inflow DOC concentrations of sewage effluent before/after chlorination and of tap water were constant at 6.0, 6.0, and 2.3 mgC l⁻¹, respectively (Figure 3). The DOC concentrations of outflow solutions fed with sewage effluent were always higher than that of tap water. All the outflow DOC concentrations were the highest on day 2, and on average, 3.5 mgC l⁻¹ greater than that of the inflow solution. Outflow DOC was the lowest on day 10. On day 28, outflow DOC concentrations of the columns fed with sewage effluents increased by 2.0 mgC l⁻¹, whereas no change in that of tap water was observed.

In experiment 2, the DOC concentrations of inflow solutions increased after disinfection. The inflow DOC concentration was, on average, 6.5 mgC l⁻¹ after chlorination and 5.8 mgC l⁻¹ after UV-disinfection, compared with 5.4 mgC l⁻¹ in the effluent before disinfection. The DOC concentration of all the outflow solutions was, on average, 20%–29% lower than that of the inflow solutions throughout the experiment, except between days 26 and 34 (Figure 4). After one week of infiltration, all the outflow DOC concentration decreased to similar levels (nondisinfected, 3.3 ± 1.0 mgC l⁻¹; UV-disinfected, 3.1 ± 0.6 mgC l⁻¹; chlorinated, 3.7 ± 0.5 mgC l⁻¹). This lasted until day 41 although the inflow DOC concentration were different (nondisinfected, 4.6 ± 1.0 mgC l⁻¹; UV-disinfected, 5.0 ± 0.9 mgC l⁻¹; chlorinated, 6.3 ± 1.4 mgC l⁻¹). From days 26 to 34, DOC removal was not obvious, but it was recovered from day 41 on.

Soil microbial community shift For all the samples in experiment 1, the relative intensities of some DGGE bands in each soil
sample changed gradually during the 4 weeks (Figure 5a). According to the band patterns summarized on the MDS map, the changes in band intensity would have reflected the number of each bacterial component in the community but not the overall soil bacterial community structure (Figure 5b). At the beginning of feeding, the soil microbial communities, including those in each inflow solution, were close to the original soil community composition. The DGGE band patterns changed when treated sewage was fed into the soil, unlike when tap water was added, but no distinct difference in band profiles between nondisinfected and chlorinated samples was observed.

In experiment 2, the soil microbial communities were transiently disturbed by UV–disinfected and chlorinated effluents particularly in the first 20 days, however, they had nearly recovered to that of day 0, by 50 days (Figure 6a and 6b). When UV–disinfected and chlorinated effluents were injected into the soil, the dissimilarity indices against day 0 on day 20 were 69 and 39, respectively. And then that of day 50 converged on 33 and 23, respectively. The effect of nondisinfected effluent on the soil microbial community was relatively small compared to disinfected ones, showing less community shift. The dissimilarity indexes between day 0 and day 20, and day 0 and day 50 were both 30.

Phylogenetic analysis of DGGE bands

Most of the 49 analyzed sequences obtained from DGGE in experiment 1, shown in Figure 7, were found to be bacteria that are generally widespread in many types of soil[27–29]. Not all the bands were sequenced, but the majority of analyzed sequences were closely related to the Holophaga/Acidobacterium group and the Proteobacteria. Furthermore, the bacterial species obtained from the bands of which the intensities increased over time were mostly affiliated with the beta subdivision of the Proteobacteria (Figure 5a and Figure 7).

DISCUSSION

The soil microbial community shifts were analyzed to show the effect of secondary treated effluent feedings on the indigenous bacterial ecology, which has the key roles in water purification for the artificial groundwater recharge system. Simultaneously, the DOC removal efficiency was monitored to see the purification ability by soil.

The DGGE patterns gradually changed in all three series of experiment 1, indicating that the soil microbial communities change by being fed with not only sewage effluent but also tap water. The feeding with tap water into the soil shows the effect of water flow on the soil microbial community, such as efflux or death of indigenous bacteria. The feeding with sewage effluents shows the effect of their chemical and biological components on the soil microbial community in addition to the effect of water flow. The difference between the effect of nondisinfected and chlorinated sewage effluent shows the effect of chlorination. The shift in the soil microbial community by being fed with sewage effluents was greater than by tap water, however, there was little difference in soil microbial communities between sewage effluent feedings treated with and without chlorination as shown in Figure 5b. These findings indicate that the effect of feeding with sewage effluents on the soil microbial community is greater than that of tap water. This also suggests that there is little effect of chlorination by-products on the soil microbial community.

In experiment 2, the feeding with three different sewage effluents shows the different
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Fig. 5 Change in microbial community in experiment 1. (a) Shift in DGGE band patterns during the 28-day-feeding of sewage effluent. The circled numbers indicate the bands that represented more than 2-point increase in proportion of band existence (Pak) and kept their Pak more than 4% on day 28. (b) MDS analysis of the DGGE band patterns of soil fed with nondisinfected sewage effluent (filled triangles), chlorinated sewage effluent (filled circles), or tap water (open squares), and of original soil (filled diamond). The increase in size of the symbols represents the different sampling points (days 0, 7, 14, and 28).
Fig. 6 Change in microbial community in experiment 2. (a) Shift in DGGE band patterns during the 50-day-feeding of sewage effluent. (b) MDS analysis of the DGGE band patterns of inflow solutions (open symbols), of soil fed with sewage effluents (filled symbols), and of original soil (filled diamond). The increase in size of the symbols and numbers represents the different sampling points (days 0, 8, 20, 30, 40, and 50). Symbols: circles, nondisinfected; squares, UV-disinfected; triangles, chlorinated.

effect of their chemical and biological components on the soil microbial community owing to the different treatment on the inflow solutions. However, every soil microbial community had nearly recovered to the band patterns observed before infiltration by 50 days. This indicates that the disinfection and its method have a transient effect on the soil microbial community shift, but all the community fed with sewage effluent can return close to the starting point by 50 days. On the other hand, the soil microbial community never approached to the one in the influents (sewage effluents). The bacteria in the influents could not increase their population in the soil.

In experiment 1 we examined the transition in DOC concentration in columns fed with two kinds of sewage effluent or tap water. Our findings indicate that the change in DOC concentration of the outflow solutions can be divided into three phases. In phase 1 soluble soil organic matter is washed out, in phase 2 organic matter in the treated sewage is trapped in the soil, and in phase 3 the soil’s capacity to accumulate and biodegrade...
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Fig. 7  Phylogenetic relationships of partial 16S rRNA gene sequences obtained from DGGE. The numbers are identical to the ones shown in Figure 5a. Circled numbers represent the sequences of DGGE bands that increased intensity during the 28-day of infiltration. The percentages of 100 bootstrap replicates are shown at each branch. This tree was established by the neighbor-joining method. An archaeral partial 16S rRNA gene sequence (*Methanococcus vannielii*, AY196675) was used as the outgroup (not shown).
organic matter is exceeded. Phase 1 corresponds to the highest outflow DOC concentration, which occurred on day 2 and exceeded the inflow DOC concentration. This excess DOC must have been soil organic matter, not DOC from the inflow solutions, because the amount of excess DOC from the columns fed with treated sewage was very close to that from the column fed with tap water. After the soil organic matter had been washed from the tap water column, the outflow DOC concentration became steady and approximately equal to the inflow DOC. In phase 2, the outflow DOC concentrations of the columns fed with either type of treated sewage—particularly nondisinfected effluent—decreased to below the inflow DOC concentrations, suggesting that organic matter in the treated sewage was trapped in the soil by adsorption or degraded. In phase 3, after day 10, the higher outflow than inflow DOC in the sewage effluent columns indicated either the dissolution of particulate organic matter trapped in the soil or an exceeding of the soil’s capacity to accumulate and biodegrade organic matter.

Likewise, in experiment 2 the decreased efficiency of removal of organic matter by the soil after day 20 indicated that the soil had exceeded its capacity to accumulate and biodegrade organic matter from days 20 to 34. However, after day 41 the soil recovered the efficiency in removing organic matter. The increased inflow DOC by disinfection—particularly by chlorination—indicates the solubilization of suspended organic matter. In spite of the different concentration of the inflow DOC, the outflow DOC concentrations of all the columns were almost equal from the beginning of infiltration to day 41.

Our overall results, particularly that of experiment 2, indicate that day 28 is the turning point of the capacity of the soil column with regard to adsorption of organic matter and community shift of the indigenous bacteria. The DOC removal efficiency decreased after day 20 but started recovering after day 41 on, as shown in Figure 4. The DGGE profile showed that the soil microbial community transiently changes owing to feeding with sewage effluent but recovers to the community structure of day 0 after day 30, as shown in Figure 6b. DOC removal efficiency recovered with the recovery of the initial soil microbial community structure, suggesting that from day 40 onward DOC removal by biological degradation compensate for the limitation of that by sorption to soil particles. From the results of DGGE and phylogenetic analysis, indigenous bacteria, which were predominated by β-Proteobacteria, would contribute to the recovery of DOC removal efficiency by biological degradation in soil.

Our findings provided insights into the tolerance for sewage effluent of the indigenous soil bacterial ecology for longer than 4 weeks. Further research on microbes that degrade problematic substances in sewage effluent will help us to maintain and improve the purification ability of soil in artificial recharge systems.

**CONCLUSIONS**

We analyzed shifts in the soil microbial community in response to feeding with secondary treated effluent of a domestic wastewater treatment plant. Its response to feeding sewage effluent was greater than tap water, whereas the disinfection method used on the effluent did not make much difference in the effect on the soil microbial community shift. The soil microbial community returned close to the community of day 0 after the 50-day of infiltration. The 30 g of soil in each column deteriorated in DOC removal by feeding sewage effluent for 28 days, but DOC removal efficiency started recovering with the recovery of soil microbial community. Our findings show that the effect of sewage effluent on a soil microbial community is small and transient, suggesting that the soil microbial community is tolerant of sewage effluent.

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