Metagenomic and functional analyses of the consequences of reduction of bacterial diversity on soil functions and bioremediation in diesel-contaminated microcosms

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The relationship between microbial biodiversity and soil function is an important issue in ecology, yet most studies have been performed in pristine ecosystems. Here, we assess the role of microbial diversity in ecological function and remediation strategies in diesel-contaminated soils. Soil microbial diversity was manipulated using a removal by dilution approach and microbial functions were determined using both metagenomic analyses and enzymatic assays. A shift from Proteobacteria- to Actinobacteria-dominant communities was observed when species diversity was reduced. Metagenomic analysis showed that a large proportion of functional gene categories were significantly altered by the reduction in biodiversity. The abundance of genes related to the nitrogen cycle was significantly reduced in the low-diversity community, impairing denitrification. In contrast, the efficiency of diesel biodegradation was increased in the low-diversity community and was further enhanced by addition of red clay as a stimulating agent. Our results suggest that the relationship between microbial diversity and ecological function involves trade-offs among ecological processes, and should not be generalized as a positive, neutral, or negative relationship.
perturbations\textsuperscript{17,18}. This discrepancy among results regarding biodiversity and community stability can perhaps be explained by the different functions investigated.

Here, we assess the roles played by microbial diversity in soil function in natural and diesel-contaminated soils. We manipulated microbial diversity using a removal by dilution approach and performed metagenomic analysis to investigate the genetic basis of a variety of processes; additionally, we performed several enzyme assays. To assess the impact of loss of biodiversity on bioremediation, we spiked red clay and processed red clay with diesel, as it is a biostimulant\textsuperscript{19}. Processed red clay is produced from red clay using a patented method (Patent# 0886082 in the Republic of Korea); it promotes diesel biodegradation and alters microbial community structure.

Results and Discussion

Bacterial abundance after dilution and soil recolonization. Bacterial communities were quantified using the 16S rRNA gene copy number as a molecular marker (Fig. 1). At week 0, the 16S rRNA gene copy number was $1.88 \pm 0.14 \times 10^{14}$ to $1.64 \pm 0.17 \times 10^{15}$/g dry soil and $5.99 \pm 0.03 \times 10^{12}$ to $6.30 \pm 0.26 \times 10^{12}$/g dry soil for the microcosms inoculated with the $10^{-2}$ and $10^{-5}$ dilutions, respectively. The difference between the 16S rRNA gene copy numbers of the $10^{-2}$- and $10^{-5}$-inoculated samples was approximately $10^3$, consistent with the difference in dilution factors. Following a transient increase at week 1, the 16S rRNA gene copy number in $10^{-2}$-inoculated samples reached approximately $1.0 \times 10^{15}$ after 6 weeks; no significant differences were observed between treatments. It should be noted that diesel-spiked samples showed a higher 16S rRNA gene copy number.

Figure 1. Schematic description of experimental design and quantitation of microbial communities in microcosms based on 16S rRNA gene copy number. (a) Soil was serially diluted and inoculated to sterilized soil slurry microcosms subjected to metagenomic analysis and experiments. (b) Copy number of the 16S rRNA gene in the $10^{-2}$-inoculated samples and (c) $10^{-5}$-inoculated samples. C, control; D, diesel-spiked soil; DR, diesel-spiked soil and red clay; DP, diesel-spiked soil and processed red clay.
that controls during incubation (Fig. 1). The increase in community size observed in diesel-spiked soil is consistent with the results of a previous study\(^\text{19}\); the diesel supplied an alternative carbon source to soil organic matter. After 6 weeks, the \(10^{-2}\) and \(10^{-3}\)-inoculated samples showed identical bacterial abundance. Different dilutions of microbial samples have previously been shown to have similar capacity to colonize sterile soil\(^\text{20}\).

**Microbial community structure differs at different levels of diversity.** The alpha diversity calculated using MG-RAST showed that, in the control treatment, the \(10^{-2}\)-inoculated samples contained a higher number of OTUs (268 OTUs) than the \(10^{-3}\)-inoculated samples (99 OTUs) (Supplementary Fig. S1). The presence of diesel in the soil further decreased the number of OTUs to 162 and 51 in the \(10^{-2}\) and \(10^{-3}\)-inoculated microcosms, respectively. The reduction in diversity produced by hydrocarbon contamination is consistent with the results of previous studies\(^\text{12}\). This could be attributed to the toxicity of diesel, as shown in studies based on single strains and communities of bacteria\(^\text{12,21}\). The soil sample used in this study was the identical sample described in our previous study\(^\text{19}\). According to the previous community analysis of soil without any treatment or incubation, *Proteobacteria* is the predominant phylum followed by *Acidobacteria* and *Actinobacteria* and their relative abundances were 50%, 13%, and 10%, respectively. Predominant families were *Rhodospirillaceae* (9%), *Comamonadaceae* (7%), and *Bradyrhizobiaceae* (7%). Serial dilution approach would have removed minor taxa comprising less than 1% relative abundance. Taxonomic analysis of the bacterial community using 16S rRNA gene sequences from the metagenomic dataset showed that *Proteobacteria* was the most abundant phylum in all 10 \(-2\)-inoculated microcosms, whereas all \(10^{-3}\)-inoculated microcosms, except the DP5 treatment, were dominated by *Actinobacteria* (Fig. 2a). In contrast, Tardy et al.\(\text{23}\), who also used a removal by dilution approach to manipulate bacterial diversity, found that the abundance of *Proteobacteria* increased with increasing dilutions. This discrepancy can be explained by differences in soil, since the abiotic properties of the soil will influence the establishment and survival of inoculated strains, known as the “habitat filtering” effect. The soil type will also determine the soil microbial seed bank used for inoculation.

In addition to its effect on the alpha diversity, diesel also appeared to affect the dominance of certain phyla, with *Proteobacteria* and *Actinobacteria* being more abundant in diesel-spiked microcosms. These taxa are commonly found in both pristine\(^\text{23}\) and diesel-contaminated soil\(^\text{12}\), and a large proportion are potentially able to degrade alkanes, a major component of diesel\(^\text{24}\). Given their prevalence and alkane-degrading capacity, the reduction in microbial diversity caused by the addition of diesel, which is toxic to many microbes, might have provided the preferred conditions for proliferation of *Proteobacteria* and *Actinobacteria\(^\text{12}\). Thus, the relative abundance of *Proteobacteria* and *Actinobacteria* increased from 70.5% and 77.3% in C2 and C5 to 95.2% and 90.6% in D2 and D5, respectively. *Actinobacteria* was the second-most abundant phylum in D2, DR2, and DP2, whereas *Bacteroidetes* was the second-most abundant group in C2. A Shift from *Proteobacteria* to *Actinobacteria*-dominant community of \(10^{-2}\) and \(10^{-3}\)-inoculated sample, respectively, was mainly due to increase in abundance of genus *Arthrobacter*, *Rhodococcus*, and Nocardioiodes which were known as hydrocarbon degraders\(^\text{25–27}\). More specifically, *Pseudomonas* and *Arthrobacter* were the predominant genera in C2 and C5, respectively (Fig. 2b). *Arthrobacter*, *Rhodococcus*, and Nocardioiodes were identified in D5. When diesel was present in red clay or processed red clay, the relative abundances of *Xanthomonas* (DR2), *Cupriavidus* (DR2 and DP2), *Phenylobacterium* (DP2), *Brevundimonas* (DP2), and *Caulobacter* (DP5) were higher than in C2. We observed increases in the relative abundance of *Brevundimonas* and *Agrobacterium* in DP2 and DP5. Processed red clay promoted the abundance of these two genera of *Alphaproteobacteria*, resulting in *Proteobacteria*-dominant communities in DP2 and DP5, regardless of the dilution factor of the initial inoculum. Community structure has often been reported to shift following hydrocarbon contamination\(^\text{12,19,28}\). The change in community profile has usually been explained by differing environmental conditions. For example, phylogenetic analysis of the bacteria present in contaminated soils showed that the dominance of a bacterial group depended on temperature and soil organic matter content\(^\text{15,28}\). However, our data demonstrate that biodiversity can also be a determining factor in shaping community structure when hydrocarbon contamination occurs.

**Changes in community diversity and structure result in differences in functional gene abundance.** The results of taxonomic binning of functional genes were consistent with the results of our community analysis based on rRNA sequences. For example, a greater proportion of *Actinobacteria*-affiliated genes were observed in the \(10^{-3}\)-inoculated samples (Supplementary Fig. S2). PCA based on phylum-level community structure (Supplementary Fig. S3A) and functional annotation of metagenomic data (KO level 3) (Supplementary Fig. S3B) showed that structurally similar communities are also functionally similar; suggesting that changes in community membership resulting from diversity manipulation also brought functional changes. The abundance of functional genes, as determined by metagenomic sequence analysis, was compared using STAMP. Of the 140 KO categories analyzed, 51, 74, 46, and 43 differed significantly for the C, D, DR, and DP treatments, respectively, between \(10^{-2}\) and \(10^{-3}\)-inoculated microcosms (Welch’s two-sided t-test, \(p<0.05\)). This linkage between function and community structure has previously been described in soil ecosystems. For example, catalytic capability was highly associated with copiotrophic and oligotrophic taxa, which dominate areas of high and low nitrogen fertilization, respectively\(^\text{29}\). We observed that some functions that are performed by all bacteria, such as amino acid metabolism and nucleotide metabolism, did not differ between samples. However, specific functions that are only performed by certain groups of bacteria, such as hydrocarbon degradation and tetracycline biosynthesis, were significantly affected by changes in community diversity and composition. Susceptibility of some functions to loss of diversity was previously reported from heavy metal-contaminated soil\(^\text{30}\); the susceptibility was attributed to the fact that these functions are performed by a narrow group of specialized species. According to the orthologue richness reduction model, loss of species richness must be <10% to avoid significant loss of ecosystem services\(^\text{28}\). Given the number of OTUs identified in the samples, \(10^{-3}\)-inoculated samples might have lost a subset of species that perform a specific ecological function. The different clustering of \(10^{-2}\)-inoculated
samples on the PCA plot suggested that the functions that remained would differ by treatment, for example between the two types of red clay contaminated with diesel.

**Effect of microbial community manipulation on alkane degradation.** To assess the effect of diversity loss on the potential of the communities present in the different soils to degrade diesel, genes related to alkane oxidation, such as those encoding alkane monooxygenase, cytochrome P450, alcohol dehydrogenase, and aldehyde dehydrogenase, were analyzed (Fig. 3). Our results showed that the reduction in microbial community diversity did not always lead to a decrease in functional gene abundance. Thus, the abundance of the alkane monooxygenase gene was 2.1–13.1-fold higher in low-diversity communities. Previous studies showed that alkB abundance generally increases in alkane-contaminated soils; however, the appearance of various primary alkane-degrading species has not been discussed in terms of the reduction of diversity. The abundance of cytochrome P450 genes did not differ significantly between treatments, except for D2 and D5. Alkane monooxygenase and cytochrome P450 were associated mainly with Actinobacteria. At the genus level, alkane 1-monooxygenase was taxonomically affiliated with Rhodococcus, Nocardioïdes, and Gordonia. Investigation of the taxonomic affiliations of the alcohol and aldehyde dehydrogenases revealed that Proteobacteria and

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**Figure 2.** Analysis of microbial communities using rRNA gene sequences and MG-RAST. Taxa with abundance <5% are presented as “Others.” (a) Phyla (b) Genera C, control; D, diesel-spiked soil; DR, diesel-spiked soil and red clay; DP, diesel-spiked soil and processed red clay.
Actinobacteria predominated in the 10⁻² and 10⁻⁵-inoculated microcosms, respectively. However, no significant changes in the abundances of alcohol dehydrogenase or aldehyde dehydrogenase genes were observed. Alcohol dehydrogenase and aldehyde dehydrogenase are expected to also be present in bacteria other than Proteobacteria or Actinobacteria because these enzymes degrade various forms of alcohols and aldehydes, respectively. The major component of the diesel used in this study was alkanes, with chain lengths of C9–C20; GC-MS analysis showed that levels of aromatic hydrocarbons and alkanes with longer chains (C21–C24) were very low. Therefore, the efficiency of diesel biodegradation was estimated based on the concentration of alkanes (C9–C20). After 6 weeks, the concentration of diesel was determined. The presence of red clay or processed red clay enhanced biodegradation of alkanes, as described previously⁹ (Fig. 4). Alkane biodegradation was more efficient in low-diversity microbial
Effect of microbial community manipulation on biogeochemical cycling. We first focused on the nitrogen cycle because nitrogen is a key nutrient that may be vulnerable to environmental perturbations, according to previous studies performed in polluted areas or experimentally manipulated conditions. We assessed how N-cycling was affected by diversity loss by analyzing the relative abundances of N-cycle-related genes from our metagenomic data. We found lower abundances of the napA, nirK, norB, and nosZ denitrification genes in the 10−2-inoculated samples than in the 10−3-inoculated samples. In contrast, the abundance of narG, which encodes the nitrate reductase that catalyzes the first step of the denitrification and DNRA (dissimilatory nitrate reduction to ammonium) processes, was greater in the 10−3-inoculated samples than in the 10−2-inoculated samples, except in the case of DP5 (Fig. 5). Most of the narG genes identified were found to belong to Actinobacteria, which supports the results of our taxonomic analyses of the bacterial community and suggests that nitrate-reducing Actinobacteria were selected during the soil recolonization process. To assess how these changes affected N cycling, the reduction of NO3− to NO2− was monitored. The concentration of nitrate decreased over time in all microcosms and D5 and DR5 showed a greater extent of nitrate reduction than D2 and DR2 (Fig. 6). Here, the experimental data only partially correspond with our metagenomic analysis of N cycle-related genes. This could be due to the fact that nitrate concentration is not only dependent on nitrate reduction by denitrification or DNRA but also on nitrate assimilation. We also investigated the effects of diversity loss on carbon, sulfur, and phosphate cycling by monitoring pectin utilization, along with β-galactosidase and arylsulfatase activities. We identified differences in pectin utilization and in β-galactosidase and arylsulfatase activities among the various microbial communities; however, we could not identify any patterns among samples. Arylsulfatase activity did not differ significantly among samples (Supplementary Fig. S4). The abundance of genes encoding pectinesterase, β-galactosidase, arylsulfatase, and phosphatase was not correlated with the activity data, probably due to non-specific enzymatic reactions and the heteropolymery properties of pectin. Although an attempt has been made to use soil enzyme activity as an indicator of hydrocarbon bioremediation, contradictory results were obtained. The absence of a general pattern in soil enzyme activity with hydrocarbon contamination and differences in diversity could be due to physiological complexity at the community level. However, our metagenomic data and enzyme activity analyses suggested that community membership, as determined by community diversity, affected the nutrient cycle in a contaminated soil environment.

Conclusion

Collectively, our results highlight that both community diversity and membership are key drivers of soil function. Thus, we showed that diesel biodegradation was enhanced in less-diverse soil microcosms due to the greater abundance of bacterial groups possessing genes involved in diesel biodegradation. However, loss of biodiversity resulted in decreased abundance of genes involved in reduction of nitrite, nitric oxide, and nitrous oxide. Genetic and experimental evidence clearly showed that biodiversity affected alkane biodegradation, denitrification, and nutrient cycling-related soil enzyme activity, in varying ways. Microbial taxa present at low abundance under natural conditions might gain a competitive advantage and thrive when initial biodiversity is impaired. Performance of specialized functions associated with a specific taxon results in a shift in the corresponding ecological function. Changes in community structure, diversity, and particular functions often occur in soils contaminated with other pollutants, such as heavy metals, and it would be revealing to implement the analyses employed in this study in various polluted environments. Many previous studies drew hasty conclusions about the relationship between ecological function and biodiversity, based on alteration of one or two functions, while neglecting to examine
others. The combination of metagenomic and enzymatic analyses performed in this study suggests that some functions might be strengthened while others were lost during shifts in the microbial community, and that such trade-offs between ecological functions were brought about by loss of diversity. This conclusion may also be relevant to the study of bioremediation, which often pursues a reduction in the total quantity of pollutants as the primary or only goal; due consideration should be given to ecological function during the bioremediation process. Overall, our results indicate that community membership should be taken into account when assessing the relationship between biodiversity and ecosystem function, highlighting process-specific responses to loss of biodiversity.

Methods
Manipulation of diversity in a diesel-contaminated microcosm supplemented with red clay.
The soil used in this study was the same as that described previously19. Briefly, soil samples were collected from Bucheon (Kyonggi province, Republic of Korea; N 37°30′26.44, E 126°48′39.01). The soil had a sandy loam texture and 30% water content. Soil pH was 9.44 ± 0.22 and contained 13,846 ± 435 mg/kg of total organic carbon. Soils were sieved to achieve a particle diameter of <2 mm. The soil slurry microcosm was generated in a 100-mL flask containing 10 g of soil and 50 mL of distilled water (DW), which was autoclaved (Fig. 1a). The soil in the microcosms was the source of carbon and other nutrients. Microcosm experiments were performed in quadruplicate. After constructing the microcosms, we extracted DNA from the soil slurry using a NucleoSpin Soil kit (Macherey-Nagel, Germany), according to the manufacturer’s instructions. Soil slurries from the microcosms were centrifuged to obtain soil particles and microbial cells. Supernatants were discarded and pellets containing soil and cells were subjected to DNA extraction. The 16S rRNA gene was amplified using 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) primers. PCR was performed at 95 °C for 90 s, followed by 35 cycles at 95 °C for 24 s, at 56 °C for 24 s, and a final extension step at 72 °C for 5 min using a Mastercycler thermal cycler (Eppendorf, Hauppauge, NY, USA).
Additionally, we assessed β-galactosidase activity in autoclaved soil samples. All soil samples were PCR-negative and lacked β-galactosidase activity, indicating that microbial activity had been abolished. Microcosms lacking red clay were spiked with 0.1% (v/v) diesel, while those with red clay were spiked with 1% (v/v) diesel. Microcosms containing processed red clay were spiked with 0.1% (v/v) diesel. All red clay was autoclaved before use. These two types of red clay were previously shown to enhance diesel bioremediation19. To manipulate biodiversity, we employed a dilution strategy that has been described previously3. Experimental procedures are depicted in Fig. 1a. Intact soil (1 g) was mixed with 10 mL of sterile DW by vortexing for 10 min. Soil suspensions were serially diluted ten-fold. We used 1 mL of the 10^{-2} and 10^{-5} diluted suspensions to inoculate the autoclaved microcosms and examined four different treatments: no treatment (control); 0.1% (v/v) diesel; 1% (v/v) diesel and red clay; and 0.1% (v/v) diesel and processed red clay. We designated the control, diesel, and diesel and red clay, and diesel and...
processed red clay groups as C, D, DR, and DP, respectively; we added a ‘2’ or ‘5’ to each group designation to indicate the dilution factor used. Microcosms were incubated at 20 °C with shaking at 150 rpm; the incubation temperature we used was within the temperature range of the sampling site.

Gas chromatographic analysis of diesel. The composition of the diesel used for spiking of soil samples was analyzed using a gas chromatography equipped with a mass spectrometer (GC-MS) (Agilent 6890N gas chromatograph and 5975 mass spectrometer). A HP-5MS column was used. Diesel dissolved in dichloromethane (2 μL) was injected into the GC. The injector and detector were maintained at 280 °C and 300 °C, respectively. Oven temperature was held at 45 °C for 2 min and increased to 310 °C at a rate of 10 °C/min with an additional hold for 25 min. For chemical identification of peaks, results were searched against the MS spectral library (NIST05 library) with a score cutoff of >80%. Quantification of residual diesel after incubation of microcosms were performed using a GC equipped with a flame ionization detector (FID), as previously described19.

Quantification of bacterial community abundance. After inoculation, we monitored changes in microbial community size by quantitative PCR (qPCR), by determining the 16S rRNA gene copy number. For this purpose, the primers 341F and 534R were used to amplify the 16S rRNA gene 44. A standard curve for 16S rRNA gene copy number has previously been constructed by our lab45. DNA extraction and PCR were performed as described above. PCR was performed using a CFX-96 PCR machine (Bio-Rad).

Metagenomic sequence analysis. For the metagenomic sequencing analysis, DNA was isolated from duplicate soil slurry samples after 6 weeks, as described above. Generation of a sequence library was conducted using the MiSeq System (Illumina Inc., San Diego, CA, USA) at Macrogen Inc. (Seoul, Republic of Korea) following the 2 × 100-bp paired-end protocol. Sequencing data was processed using Illumina Pipeline (CASAVA) version 1.8.2. Paired-end reads that contained >80% of bases with a base quality ≥ Q16 were assembled. The results of sequencing are summarized in Supplementary Table S1. Because the depth of sequencing differed between samples, assembled sequences were subsampled (5,375,854 reads, the minimum number of reads) and uploaded to MG-RAST46 for further analysis. Metagenomic sequences were deposited in MG-RAST (http://metagenomics.anl.gov/) under deposition numbers 4569530.3 to 4569537.3. Statistical analysis of the metagenomic
sequencing data was performed using STAMP\textsuperscript{47} and R. Community structure was analyzed based on ribosomal RNA sequences from the metagenomic data using the Best Hit Classification function and the MSRA database available in MG-RAST. To identify the taxonomic affiliation of functional genes, selected genes were stored in Workbench and then subjected to Best Hit Classification using the M5NR database. The cutoff for metagenomic analysis was a maximum e-value of 1e-5, a minimum identity of 60\%, and a minimum alignment length of 15 bp.

**Measurement of inorganic nitrogen compounds.** Another set of microcosms were used to monitor concentrations of nitrates and nitrites. These microcosms contained autoclaved soil slurry and were inoculated from microcosms that had been incubating at 20\°C for 6 weeks. The caps of the bottles were tightly sealed and helium gas was used to replace the gas in the bottle headspace. The concentrations of nitrites and nitrites in the soil slurry were measured using an ion chromatograph equipped with an autosampler ( Dionex AS40, USA). The mobile phase (DW) passed through the column at a flow rate of 1.0 mL/min. The sample injection volume was 5 mL. The suppressor was generated using 20 mM potassium hydroxide. A standard curve was established using nitrate and nitrite standard solutions (Kanto Chemical, Japan).

**Assays for determining various ecological functions.** After 6 weeks, soil slurry microcosms were tested to identify differences in various ecological functions. To determine their ability to utilize pectin, soil slurry samples were transferred to sterile minimal salt basal media\textsuperscript{48} supplemented with pectin. Utilization of pectin was assessed through the determination of increased protein content after 1 week. We determined the activities of \(\beta\)-galactosidase, phosphatase, and aroylsulfatase as described previously\textsuperscript{49–51}.

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