BIOLOGICAL IMPACT ON THE DEGRADATION OF 1,4-DIOXANE AND 4-CHLOROPHENOL IN RIVERWATER

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Abstract
1,4-Dioxane and chlorophenols are pollutants of concern in riverwater and even in groundwater due to their wide presence and probable carcinogenicity. Therefore it is essential to understand their environmental fate and degradation pathways by microorganisms in an aquatic environment. In this study, we evaluated the natural degradability of 1,4-dioxane and 4-chlorophenol in riverwater which was already analyzed for bacterial community through denaturing gradient gel electrophoresis (DGGE). Pollutants (10 ppm) were mixed with riverwater and aerobically incubated for 25 days. For comparison, the 1,4-dioxane degrading actinomycete, *Pseudonocardia dioxanivorans* ($CB1190^T$) was artificially employed at the different conditions using 1,4-dioxane as the carbon source. The initial concentrations of 1,4-dioxane and 4-chlorophenol were slightly decreased in the natural condition over 25 days, however, this only showed these pollutants are considerably non-degradable compounds. While *P. dioxanivorans* degraded 1000 ppm of 1.4-dioxane completely as a pure culture with minimum salt medium, direct bacterial inoculation to riverwater did not significantly enhance the degradation rate. Therefore, several nutritional deficiencies or bacterial competition might be the reason for these results.

Introduction
1,4-dioxane is generally used industrially as a stabilizing solvent for producing some chlorinated chemicals or textiles. Despite their effectiveness for industrial uses, its low K_{ow} value and Henry's Law constant made this chemical difficult to remove or destroy by traditional treatment processes in the discharged wastewater.[1] The most applicable technology is the photoremediation by UV light or ozonic destruction in the presence with hydrogen peroxide, however, they are still highly costly approaches.[2,3] Also chlorophenols are widely used in many industrial applications and enter the environment through accidental spillage and/or because of inappropriate management controls. Therefore, they are also found in soil, sediments, surface water, and wastewater, and are listed as priority environmental pollutants by the US EPA because of their toxicity.[4]
Due to the ubiquitous presence of these chemicals in the riverwater, their fate and monitoring are extensively being investigated in the aquatic environment. Several physical and/or chemical treatments have been attempted, economical inefficiency and difficulties to *in situ* application are still remained as problems yet.[5] Although a large number of biological activities have also been identified to degrade or transform the toxic xenobiotics, we are still attempting to scale lab batch treatment up to *in situ* applications for these compounds. Therefore this research analyzed microbial consortium in riverwater and tested natural degradabilities of 1,4-dioxane and 4-chlorophenol at the 10 ppm concentration. For comparison, the 1,4-dioxane degrading actinomycete, *Pseudonocardia dioxanivorans* ($CB1190^T$) was artificially employed at the different conditions using 1,4-dioxane as a carbon source, and its effects were monitored.[6]

Materials and Methods
DGGE analysis. 4 liters of riverwater sample were taken from 4 different points along the Nakdong river in Korea, and then 500 ml of water samples was filtered using a 0.45 μm filter membrane to collect microorganisms. Bacterial cells on the filter were destroyed and chromosomal DNA was purified by a Powersoil DNA isolation kit (MoBio, Carlsburg, CA, USA). The isolated DNA was mixed with polymerase chain reaction (PCR) Premix reagent (Bioneer, Daejeon, Korea) with 338F GC-clamp primer and 907R primer and amplified by Mycycler thermocycler (Bio-Rad, Hercules, CA, USA). The amplified DNA was loaded into a DGGE Dcode system (Bio-Rad, Hercules, CA, USA) and samples were separated depending on the GC content and thermal stability.[7] After DNA separation, the agarose gel was stained by SyBr Green dye and then each band was cut under UV illumination. DNA in each cut was sequenced and then identified using NCBI Blast. Sequence alignment was achieved by ClustalX (V.1.83) and finally the phylogenic tree was drawn according to the genetic relations.
Evaluation of biodegradation. 10 ml of riverwater was stored in triplicate sets of 100 ml Erlenmeyer flasks with

10 ppm of 1,4-dioxane and 4-chlorophenol. These flasks were incubated for 25 days in a darkened shaking incubator (160 rpm, 28℃), and subsequently flasks were frozen at 5 day sampling intervals. Finally, all flasks were thawed at room temperature. Then filtered samples were analyzed by HPLC (1100 series, Agilent, Palo Alto, CA, USA). All chromatograms were obtained at 200 nm for 1,4-dioxane and 245nm for 4-chlorophenol.[8] A LC/MS/MS system (API 2000, Applied Biosystems, Foster City, CA, USA) and Polaris Q GC/MS (Thermo-Finnigan, San Jose, CA, USA) were also applied to detect some natural metabolites from the incubation. A well investigated bacterial strain *Pseudonocardia dioxanivorans* ($CB1190^T$) was obtained from ATCC (American Type Culture Collection, USA). This strain was tested to evaluate degradability of 1000 ppm of 1,4-dioxane in the minimum salts medium. To compare with natural degradation tests, 10 µl of pre-grown cell cultures was transferred to riverwater which was adjusted to 10 ppm of 1,4-dioxane, and then incubated according to the same procedures above.

Results and Discussion

Four sampling sites were selected and the microbial consortium in riverwater samples was analyzed from July and October 2006. Community analysis revealed that various bacteria existed and, in particular, the *Ralstonia* species were identified as dominant members from many samples; suggesting that they might play an important role in the degradation of pollutants. Many previous studies reported that several *Ralstonia* strains are efficient degraders of halogenated phenolic compounds. DGGE profiles of riverwater bacterial communities are shown in Fig. 1. All separated DNA bands were sequenced and their results were spatially rearranged according to their relative sequence homology. Finally, processed results were shown as a phylogenic tree of the bacterial community from the riverwater (Fig. 2).

We evaluated the natural degradability in the river water using 10 ppm of 4-chlorophenol and 1,4-dioxane as pollutants for 25 days. The concentrations of 1,4-dioxane and 4-chlorophenol were slightly decreased to 9.4 ppm and 8.6 ppm after 25 days (Fig 3 c,d); this phenomenon could be explained by occurrence of microbial adsorption or enzymatic oxidation in contrast to sterilized control experiment. However, these results proved repeatedly that these compounds are non-biodegradable and persistent.

Additionally, the well-investigated bacterial strain, *Pseudonocardia dioxanivorans* was employed for biodegradation of 1,4-dioxane in the riverwater. This strain degraded 1000 ppm of 1,4-dioxane in the minimal salts medium (Fig. 3a), however, they only showed slight degradation of 1,4-dioxane at a similar level to the natural degradation when it was inoculated into the non-sterilized riverwater (Fig. 3b). Therefore, several nutritional deficiency or bacterial competition was considered to be a reason for that phenomenon. That might have originated from the complex bacterial community structure and could affect initial degradation steps.

With concentrated river water, we attempted to detect major intermediates *ex situ*; however, due to trace amount of existing chemicals we were unable to find significant metabolized chemicals from GC/MS and LC/MS analysis. Even at 10 ppm experiments, the intermediates were not found at detectable levels.

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Fig. 1. Denaturing gradient gel electrophoresis of 4 riverwater samples from July (1) and October (2). (A) Gumi, (B) Waegwan, (C) Daegu, (D) Busan.

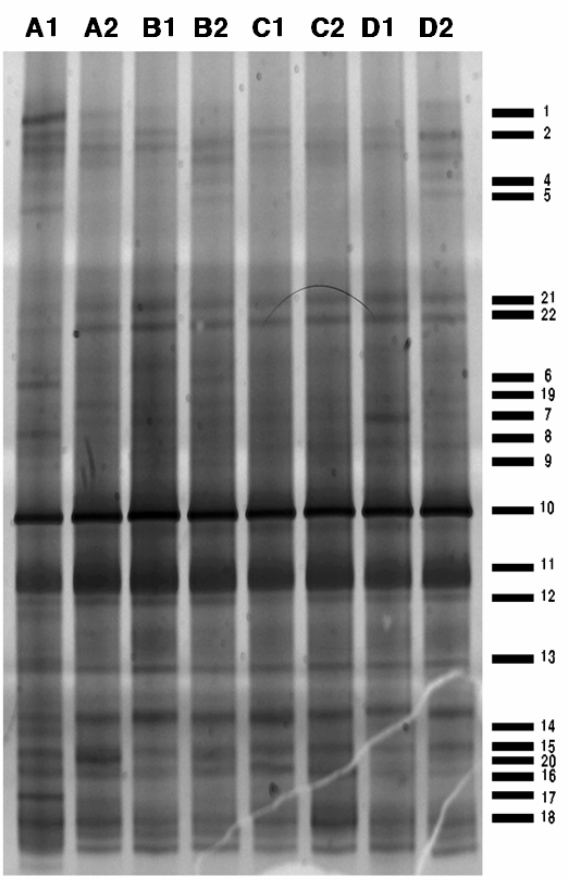

Fig. 2. Phylogenic tree of identified bacteria in riverwater through 16S rRNA PCR-DGGE analysis.

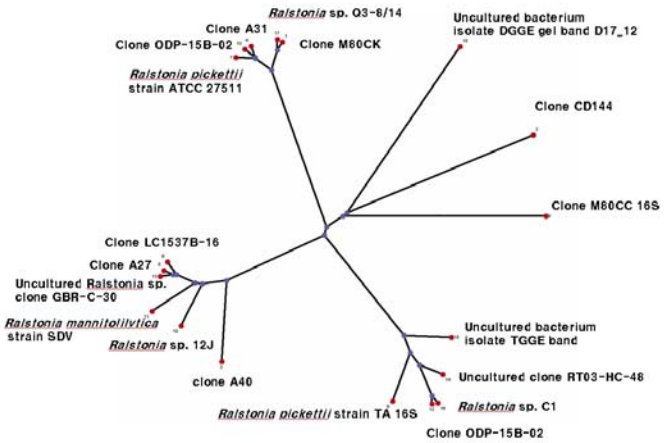

Fig. 3. Biodegradation results of 1,4-dioxane and 4-chlorophenol. (A) 1000 ppm of 1,4-dioxane in the minimal salts medium with *Pseudonocardia dioxanivorans*, (B) Inoculation of *P. dioxanivorans* to 1000 ppm of 1,4-dioxane in riverwater, (C) 10 ppm of 1,4-dioxane in riverwater, (D) 10 ppm of 4-chlorophenol in riverwater.