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Effects of 3,5-dichlorophenol on excess biomass reduction and bacterial community dynamics in activated sludge as revealed by a polyphasic approach

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The effects of 3,5-dichlorophenol (DCP) on excess sludge reduction and microbial community dynamics were studied using laboratory-scale activated sludge reactors. The addition of 3,5-DCP at an interval of 7–8 days of operation resulted in effective reduction of growing biomass without a significant decrease in substrate removal activity. However, this uncoupling effect completely disappeared after 30 days of operation. Quinone profiling showed that a drastic component shift from ubiquinone-8 (Q-8) to Q-10 as the major homolog took place during this period of operation, suggesting that Q-10-containing bacteria, i.e., *Alphaproteobacteria*, became predominant at the uncoupler-ineffective stage. This result was supported by PCR-aided denaturating gradient gel electrophoresis and clone library analyses of 16S rRNA genes and fluorescence *in situ* hybridization. Among the gene clones detected, those corresponding to *Brevundimonas* predominated at the uncoupler-ineffective stage. The uncoupler-added reactor yielded 3,5-DCP-resistant *Pseudomonas* strains as the predominant cultivable bacteria and non-3,5-DCP-resistant *Brevundimonas* strains as the second most abundant isolates These results suggest that the disappearance of the uncoupling function of 3,5-DCP during the long-term operation of the reactor is related to the drastic community change with increasing populations of *Alphaproteobacteria*. Most of these alphaproteobacteria represented by *Brevundimonas* are not resistant to 3,5-DCP but, by an unknown mechanism, may support the bioprotection of the microbial community from the uncoupling effect.

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[**Key words:** Sludge reduction; 3,5-Dichlorophenol; Uncoupler; Bacterial community dynamics; Polyphasic approach]

Reduction of excess sludge in activated sludge processes is central to research in biological wastewater treatment technology. A number of promising strategies for minimizing excess sludge, including chemical methods based on uncoupling metabolism, have been well documented (1–4). While metabolic uncouplers at appropriate concentrations have little or no effects on respiratory electron transport activity of microorganisms, they can diminish the transmembrane electrochemical gradient essential for oxidative phosphorylation and the resultant growth. Therefore, uncoupler dosing exerts the influence on activated sludge to reduce biomass as a whole in a state of keeping substrate removal performance. Representatives of chemical uncouplers which have been studied for this purpose are 3,3′,4′,5-tetrachlorosalicylanilide (TCS), nitrophenols and chlorophenols.

A previous study showed that the inhibitory effect of 4-nitorophenol (4-NP) on biomass production in an activated sludge reactor weakened gradually with time and almost disappeared after 4 weeks of operation (5). In this process, a significant change in microbial community structure was demonstrated by a biomarker method, quinone profiling, and a population shift to the 4-NP-resistant populations might be a mechanism causing the disappearance of the uncoupling effect. It has been shown that functional resistance of activated sludge following toxic-shock-loading with 4-NP and 3,5-dichlorophenol (3,5-DCP) is promoted by diversification of the predominant bacteria (6). On the other hand, the addition of 2,4-DCP to an activated sludge process caused little variation of microbial community structure (7). The addition of 2,6-DCP to an activated sludge process was useful to reduce sludge biomass, but this effect disappeared during long-term operation (8). This phenomenon might be correlated to the presence of extracellular polymer substance (EPS) whose matrix served as a protective barrier for the bacteria inside sludge flocs to 2,6-DCP (8). The EPS matrix of activated sludge has been reported to function as a protective barrier for bacteria inside sludge flocs to various toxic chemicals (9). In the case of TCS-added reactors, not only cell lysis was enhanced but also the production of EPS was stimulated (10). Increasing amounts of EPS in response to dosing with TCS might imply that microorganisms consumed more energy to resist the uncoupler (11).

Although the chemical methods based on uncoupler metabolism for excess sludge reduction have been extensively studied as noted above, information about factors affecting the effectiveness of uncoupler dosage is still fragmentary. This study was undertaken to examine time-dependent changes in uncoupling effects on excess sludge reduction during long-term operation of a laboratory sequencing-batch reactor (SBR). For this, we used 3,5-DCP as the uncoupler, because the 3-, and 5-chlorinated form may have the most toxic effect on microorganisms among dichlorophenols

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(12,13). The main purpose of the study was to elucidate how a microbial community change is involved in the time-dependent disappearance of uncoupling effects on the reactor. To address this subject, we took a polyphasic approach to community analysis by a combination of culture-independent quinone profiling, 16S rRNA gene-targeted PCR and denaturating gradient gel electrophoresis (PCR-DGGE), clone library sequencing and fluorescence *in situ* hybridization (FISH). Cultivable bacteria from 3,5-DCP-added reactors were also isolated, phylogenetically identified and characterized. Herein, we report that the uncoupling function of 3,5-DCP becomes ineffective by a long-term operation of the reactor, in which a drastic community change with increasing populations of *Alphaproteobacteria* is involved.

MATERIALS AND METHODS

Construction and operation of activated sludge reactors CULSTIR flasks (Shibata Scientific Technology, Soka, Japan) that had a working volume of 1 L were used to construct laboratory SBRs. Parent SBRs (pSBRs) were seeded with activated sludge (designated ASR0) taken from a municipal sewage treatment plant in Toyohashi, Japan, to have an initial concentration of mixed liquor suspended solids (MLSS) as 3000 mg L^{-1} and cultivated at 25°C with synthetic sewage A (SSA) (14) with 24 h batch cycles. These reactors were rotated on a magnetic stirrer at 160 rpm and aerated with an air pump to keep a dissolved oxygen (DO) tension of 1–2 mg L^{-1}. At the end of every batch cycle, half of the supernatant was exchanged with diluted SSA to make a BOD loading-rate of 1200 mg L^{-1} d^{-1} and the MLSS was adjusted to 3000 mg L^{-1}. After 6 months of operation, the sludge from pSBRs (ASR180) was introduced to two sub-reactors which were then cultivated for 70 days as for pSBRs. One of the reactors (designated SASR1) was operated as the control without 3,5-DCP dosing and the other (designated SASR2) was dosed with 150 μM 3,5-DCP as the final concentration at an interval of 7–8 days. The operation of the sub-reactors was modified by adjusting MLSS every period of 3,5-DCP dosing; that is, the excess sludge biomass was not removed from the reactors during a one-dosing period. Mixed liquor samples were taken from the reactors at the end of batch cycles and immediately used for analyses or stored at −20°C until used.

Batch tests of growth and redox activity in response to 3,5-DCP Mixed liquor samples were taken from a pSBR on day 90 and subjected to batch tests for growth and tetrazolium reduction in response to the addition of 3,5-DCP. Sludge biomass was harvested from 10 mL of the mixed liquor by centrifugation at 8000 ×*g*, washed with phosphate-buffered saline (PBS, pH 7.0), and then introduced into 100-mL Erlenmeyer flasks containing 10 mL of diluted SSA medium having a BOD of 1200 mg L^{-1}, to which 25–450 μM 3,5-DCP was added. Then, the flasks were incubated at 25°C for 24 h on a reciprocal shaker at 160 rpm, and the sludge concentration was estimated by measuring the optical density at 660 nm (OD_{660}). Tetrazolium reduction activity of sludge was measured using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT) (Sigma–Aldrich, St. Louis, MO, USA) and the electron-coupling agent phenazine methosulfate according to the protocol described by McCluskey et al. (15). The reaction mixture was incubated at 25°C for 3 h, and the relative amount of the soluble red formazan produced was estimated by measuring the absorbance at 465 nm using a Shimadzu Biospec-1600 spectrophotometer.

Continuous mixed culture with 3,5-DCP A 2-L Erlenmeyer flask (working volume, 1000 mL) equipped with a butyl rubber stopper into which flow and aeration tubes were put was used to construct a continuous culture system. The flask was seeded with the acclimated sludge from a pSBR to give an initial MLSS of 3000 mg L^{-1} and fed with 150 μM 3,5-DCP-containing SSA medium at a flow rate of 200 mL d^{-1} using PERISTA pumps (Atto, Tokyo, Japan). The reactor was agitated with a magnetic stirrer, aerated with an air pump and incubated at 25°C for 8 weeks. Mixed effluent samples were taken at appropriate intervals of cultivation and stored at −20°C until analysis.

Physicochemical analyses For measurement of MLSS, sludge samples taken from the reactors were dispersed by sonication (20 kHz; output power, 50 W) on ice or 1.5 min and measured for OD_{660}. MLSS was determined routinely using a linear regression equation showing the relationship between OD_{660} and dry weight of sludge as measured by a standard method (16). The pH of the mixed liquor of the reactors was measured with a Horiba pH meter. Dissolved oxygen (DO) tension was measured with a DO meter. For dissolved organic carbon (DOC) and 3,5-DCP measurement, samples were centrifuged at 12,600 ×*g* for 10 min. Then, the resultant supernatant was saved, filtered through membrane filter (pore size, 0.2 μm), and immediately analyzed or stored at −20°C until analysis. The concentration of DOC was measured using a Shimadzu model TOC-500 TOC analyzer (Shimadzu, Kyoto, Japan). 3,5-DCP was separated and quantified by reverse-phase HPLC as described previously (17).

Quinone analysis For quinone analysis, sludge samples were taken from pSBRs on day 0 and 180, SASR1 on days 42 and 70 and SASR2 on days 14, 28, 42 and 70. Quinones from sludge samples were extracted with a chloroform-methanol mixture, fractionated into the menaquinone and ubiquinone fractions and identified by reverse-phase HPLC with photodiode array detection as described previously (18,19). In this report, ubiquinones, menaquinones, and plastoquinones with *n* isoprene units in each side chain were abbreviated as Q-*n*, MK-*n* and PQ-*n*, respectively. Partially hydrogenated menaquinones were expressed as MK-*n*(H_x), where *x* indicated the number of hydrogen atoms saturating the side chain. Differences in quinone profiles were evaluated using the dissimilarity index *D*, a modification of city-block distance, and the microbial divergence index MD_q (18,20). Multi-dimensional scaling (MDS) (21,22) of the *D* matrix data was performed using the XLSTAT program (Addinsoft, New York, NY, USA).

Enumeration, isolation and identification of bacteria Direct total bacterial counts were measured by staining with SYBR Green I (Thermo Fisher Scientific, Waltham, MA, USA) and by observing under an Olympus model BX-50 epifluorescence microscope equipped with a DP-70 digital CCD camera (Olympus, Tokyo, Japan) as described previously (23). Viable bacteria were enumerated by plate counting using ASC agar medium (23) which was incubated at 25°C for 2 weeks before counting colony-forming units (CFUs). The enumeration media were supplemented with 100–700 μM 3,5-DCP when used to detect 3,5-DCP-resistant CFUs. Colonies from the enumeration plates were purified by streaking of plates, and 104 strains thus obtained were preserved onto PBY agar slants (24). The isolates were phylogenetically identified by 16S rRNA gene sequencing as described below.

Testing for 3,5-DCP resistance Resistance to 3,5-DCP was determined by measuring growth (OD_{660}) in PBY liquid medium supplemented with 100–700 μM 3,5-DCP and by monitoring respiratory oxygen consumption (23) in response to stepwise dosing with 400 μM 3,5-DCP in addition to 10 mM glucose.

FISH analysis Sludge biomass was collected from SASR2 on day 70 (SAS2-d70), fixed with 50% ethanol, and stored at −20°C until analyzed. For FISH assays, three oligonucleotide probes that were commercially synthesized with Alexa Fluor 546 labeling at 5′-terminus (Thermo Fisher Scientific) were used. These were ALF968 (25), BET42a and GAM42a (26), which were previously designed for specific detection of ribosomal RNA of *Alpha-*, *Beta-* and *Gammaproteobacteria*, respectively. Hybridization was performed under optimized conditions using standard FISH protocols as described (25,26). FISH stained biomass were counterstained with SYBR Green I and observed under the Olympus epifluorescence microscope system as noted above. FISH images were taken and analyzed using the ImageJ version 1.47 program (http://rsb.info.nih.gov/ij/).

DNA extraction and purification Bulk DNA of biomass from SASR1, SASR2 and bacterial cultures was extracted as previously described (27,28). Extracted DNA was further purified by a standard procedure including phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and RNase A treatment and ethanol precipitation (29).

PCR-DGGE DNA samples extracted from sludges in SASR1 and SASR2 on days 0, 14, 28, 42, 56 and 70 and from the 3,5-DCP-loaded continuous culture system were subjected to PCR-DGGE. The variable region V3 of bacterial 16S rRNA genes, corresponding to positions 341–534 in the *Escherichia coli* 16S rRNA numbering system, was PCR-amplified using the forward primer GC341f with a GC-clamp on the 5′ terminus and the reverse primer 534r (30), an AmpliTaq Gold *Taq* DNA polymerase kit (Thermo Fisher Scientific), and a Takara Thermal Cycler (Takara Bio, Otsu, Japan). The PCR profile consisted of 10 min activation of the polymerase at 94°C and 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 53°C and 1 min extension at 72°C, followed by 5 min extension at 72°C. Amplicons were purified with an UltraClean PCR Clean-up kit (Funakoshi, Tokyo, Japan) and analyzed by DGGE using a Bio-Rad DCode system (Bio-Rad, Hercules, CA, USA) as described previously (31). DGGE bands were detected by staining with ethidium bromide, photographed, and analyzed for their intensity using the ImageJ program. DNA fragments from major DGGE bands were extracted and purified for sequencing as described (31).

16S rRNA gene amplification and clone library construction Bacterial 16S rRNA gene fragments from purified DNA from the SASR2-d70 and bacterial cultures were amplified using a PCR primer set of 27f and 1492r as described previously (32). PCR amplicons from the SASR2 sludge were purified as described above and subcloned using a pGEM-T Easy Vector Systems (Promega, Madison, WI, USA) and *E. coli* JM109 competent cells according to the protocols specified by the manufacturer. Plasmid DNA was purified using GenElute HP Plasmid Kits (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. 16S rRNA gene clones as plasmid inserts were re-amplified with PCR primers 27f/1492r, digested with the restriction enzymes HaeIII, HhaI or Sau3AI, and classified into different operational taxonomic units (OTUs) based on restriction fragment length polymorphism (RFLP) patterns on agarose gel electrophoresis as described (32).

Sequencing and phylogenetic analyses of 16S rRNA genes Sequencing of 16S rRNA gene clones was performed using a BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific) and an Applied Biosystems 3100xl genetic analyzer according to the manufacturer's instructions. Sequence data were compiled with the GENETYX-MAC ver. 17 program (GENETYX Corporation, Tokyo, Japan) and compared to those available from public database using the BLAST search system (33) and the RDP Seqmatch with the type-strain option (34). Chimeric sequences

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