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Toxicological evaluation for bioremediation processes of TNT-contaminated soil by *Salmonella* mutagenicity assay

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Abstract−This research was performed to evaluate the toxicity for composting and slurry phase bioreactor processes of TNT (2,4,6-trinitrotoluene)-contaminated soils by *Salmonella* mutagenicity assay. For composting, the percentage reductions of final composts in strain TA98 and TA100 with S9 metabolic activation were 90.3-93.7% and 96.7-97.5%, respectively. For slurry phase bioreactor processes, the percentage reductions of final residuals in strain TA98 and TA100 with S9 metabolic activation were 95.0% and 99.1% for anaerobic, 96.2% and 99.2% for anaerobic/aerobic, and 96.6 and 97.4% for anaerobic treatment. Slurry phase treatment showed higher mutagenicity reduction than composting. It was implied that slurry phase treatment was a more effective process than composting in reducing toxicity. This research has the advantage of speed and ease of performance in comparison to testing of other higher life forms due to the shorter life cycles of microorganisms.

Key words: TNT, Toxicity, Composting, Slurry Phase Bioreactor, Mutagenicity

INTRODUCTION

The widespread use of organic chemicals and their release into the ecosphere has caused concern because of their toxic effects, even at very low concentrations. The toxic effects of these contaminants are manifested in groundwater as well as soil by affecting the microbial population [1]. The worldwide annual production of TNT (2,4,6-trinitrotoluene), the primary explosive used in the manufacture of munitions, is estimated at two million pounds [2]. TNT is the major explosive used by the military because of its stability, low sensitivity to impact, friction and high temperature, and well developed methods of manufacture. Also, it is widely used in commercial explosives as a good sensitizer and is much safer in production and handling than nitroglycerine [3]. TNT contamination of soil and water occurs because of the manufacture, loading, assembly, packing and military related activities [4,5]. Soil and water contaminated with explosives are primarily located on military properties including bases, ranges, and munitions manufacturing centers, both in the USA and throughout the world. These properties have been systematically contaminated over a period of more than 100 years [6]. The DoD (United States Department of Defense) has identified more than 1,000 sites contaminated with explosives, of which over 95% are contaminated with TNT and 87% exceed permissible groundwater contaminant levels [7].

TNT and its metabolites are known to be toxic to bacteria, aquatic and terrestrial plants, invertebrates, terrestrial mammals and mammalian cells, and have also demonstrated some genotoxic effects [8,9]. TNT is also toxic to a number of organisms including humans and may be carcinogenic. Because of its toxic and recalcitrant properties, the contamination of soil and groundwater by TNT represents a significant international environmental problem [4]. Due to the potential adverse effect of the compound on human health and on the environment, contaminated environments must be remediated [10].

The current emergence of different bioremediation technologies to clean up contaminated sites has aroused interest in determining concentration ranges within which microorganisms can break down the contaminants. The objective of any remediation process is to reduce the concentration of the contaminant so as to substantially eliminate the toxic effects on the environment. Although preliminary data on potential toxicity may be obtained from the available literature, it is imperative that direct toxicity testing be done to assess the problem at hand prior to and subsequent to remediation. The determination of toxicity is one of the essential features in the evaluation of possible remedial alternatives [11]. Griest et al. [12] observed 98% transformation of TNT during composting, but the compost retained about 12% of its original mutagenicity, and the aqueous leachate still had about 10% of its toxicity to an aquatic invertebrate.

Toxicological evaluations of sites contaminated with hazardous wastes have been conducted using a battery of toxicity assays: luminescent bacteria assay, Ames assay, cucumber radish, mammalian cell lines and earthworm toxicity assays. However, there are relatively few studies that describe bioremediation processes of TNT-contaminated soils in which the toxicological changes associated with bioprocess have been monitored [12-15]. In many instances, the information on kinetics comes only from evaluations of the loss of the parent molecule. This is warranted for toxicants whose inhibitory effects are totally destroyed (e.g., complete mineralization) as a result of the first enzymatic reaction or in metabolic sequences in

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which intermediates do not accumulate. It is not warranted if intermediates accumulate, especially if they are toxic or their hazard has yet to be evaluated [16]. Therefore, this research was performed to evaluate the toxicity for the ex-situ bioremediation processes of TNT-contaminated soils by *salmonella* mutagenicity assay. Composting and slurry phase bioreactor were used for bioremediation processes to treat TNT-contaminated soil.

MATERIALS AND METHODS

1. Materials

The materials for composting were soil, sewage sludge, TNT, and glucose or acetone. The soil collected from K university campus was sieved through a 2 mm sieve to remove large soil fraction such as stone and gravel. The soil texture was classified as loamy sand. Dewatered sewage sludge from Nanji Composting Facility at Nanji publicly owned treatment works in Korea was used to supply energy and nutrient sources in contaminated soils. Military-grade TNT with 76% of purity (wet weight basis) was used for this research. Glucose or acetone was selected to control C/N ratio in this research.

The materials for slurry phase bioreactor process were as follows. The same soil in composting experiment was ground with mortar and pestle to obtain finer fraction. The texture of the ground soil was a typical loam. The same military-grade TNT was used for target contaminant and molasses was used as co-substrate.

2. Experimental Condition

The experimental apparatus for composting process was referred to In et al. [17]. The composting system consisted of a 3 L-reactor, two CO_2 removal traps, a humidifier, and a trap for collecting CO_2 evolved from biodegradation. The composting reactor was placed in an incubator in which temperature was maintained at 30 °C to minimize the effect of exterior temperature variation. Aeration rate of 200 L/min·m^3 of soil would be introduced into the reactor. TNT as a target contaminant was spiked at about 1,300 mg/kg of soil on dry weight basis without inhibition to microbial population. The mixing ratio of contaminated soil to sewage sludge was set to 1 : 0.5 as wet weight basis. The optimal C/N ratios for different composting materials ranged from 20 to 35 [18]. The adapted C/N ratio was 26, which corresponded to approximately four times of the case without glucose or acetone. The composting was operated for 45 days.

The experimental apparatus for slurry phase bioreactor process was referred to In et al. [19]. TNT as a target contaminant was spiked at 1,000 mg TNT/kg soil. Different metabolic regime experiments were performed with anaerobic, anaerobic/aerobic, and aerobic reactors. To prevent photolysis of the added TNT, the reactors were incubated in the dark and operated at 30 °C. In the anaerobic/aerobic reactor, the anaerobic stage was maintained for 60 days and then transferred to the aerobic reactor that was operated until 200 days. Molasses of 0.3% (w/v) as co-substrate was added to the slurry based on total solid in all reactors. Soil loading was 30% (w/v) slurry of TNT-contaminated soil in water. The phosphate buffer adjusted to neutral pH with KH_2PO_4 and K_2HPO_4 was added to the slurry in 50 mM.

3. Analysis

To analyze TNT in solid phase composting and slurry phase bioreactor, analytical mix standards purchased from AccuStandard (Mix A (M8330A-R-10X) and Mix B (M8330B-R2-10X), New Haven, USA) were used. The standards were diluted in acetonitrile to achieve a 10 ppm. A 2.0 g sample was placed in a 10 mL glass vial equipped with a Teflon-lined cap, and 10 mL of acetonitrile was added in the vial. The target compounds in sample were dispersed by vortex mixing for 1 min and extracted in an ultrasonic bath (Branson Ultrasonic Corp., 8210R-DTM) for 18 hours. The vials were removed from the bath and allowed to settle for 30 min. A 5 mL aliquot was removed, placed in a glass vial and combined with 5 mL aqueous $CaCl_2$ (5 g/L). The vials were shaken and centrifuged at 2,000 g for 20 min. A 5 mL aliquot was filtered through a 0.45 μm PTFE filter (Millipore, JHWP02500) into a clean vial. The sample extracts were analyzed using RP-HPLC (reversed-phase high performance liquid chromatography) method (SW-846 Method 8330A) described by USEPA [20].

Salmonella mutagenicity assay (Ames assay) was performed as described by Maron and Ames [21]. *S. typhimurium* strains TA98 (frame-shift mutant) and TA100 (base-pair substitution mutant) were purchased from Molecular Toxicology Inc. (USA). Each strain was tested for its genetic traits such as histidine requirement, deep rough (rfa) characteristic, UV sensitivity (uvrB mutation), ampicillin- or tetracycline-resistance by R-factor and the number of spontaneous revertants before use.

The test was performed with and without metabolic activation (S9 mix). S9 mix was purchased from Oriental Yeast Co. (Japan). Each dilution in dimethylsulfoxide (DMSO) was performed with and without metabolic activation. For metabolic activation, preincubation was provided for 30 minutes. The positive controls were 4-nitroquinoline-1-oxide (4NQO, 0.5 μg/plate) for both strains without S9 metabolic activation and 2-aminoanthracene (2-AA, 0.4 μg/plate) for both strains with S9 metabolic activation. DMSO was tested for negative control. The number of revertant colonies was counted after 48 hours of incubation at 37 °C. According to Maron and Ames [21], a mutagenic effect was acknowledged as positive when the number of revertant colonies is more than double compared with the negative control and is dose-dependent.

RESULTS AND DISCUSSION

1. Removal of TNT Concentration for Bioremediation Processes

The composting process was operated for 45 days. In glucose C/N ratio controlled composting, TNT concentration decreased sharply until 20 days from initial 1,294.0 mg/kg to 147.5 mg/kg (Fig. 1(a)). After that time, TNT concentration slowly decreased to 106.1 mg/kg at the end of composting, which indicated a degradation efficiency of about 92%. On the other hand, in acetone added composting, TNT concentration decreased until 20 days from initial 1,302.4 mg/kg to 156.7 mg/kg. TNT concentration of the final compost was 51.5 mg/kg. The TNT degradation efficiency showed about 96%, which was slightly higher than that of glucose C/N ratio controlled experiment [17]. Radtke et al. [22] reported that acetone-pretreated soil responded to composting significantly better than untreated soil because TNT is extremely soluble in acetone, of which solubility is 130 g/100 g of acetone.

Slurry phase bioreactor operation was performed under different metabolic regimes (Fig. 1(b)). TNT concentration of anaerobic/aero-

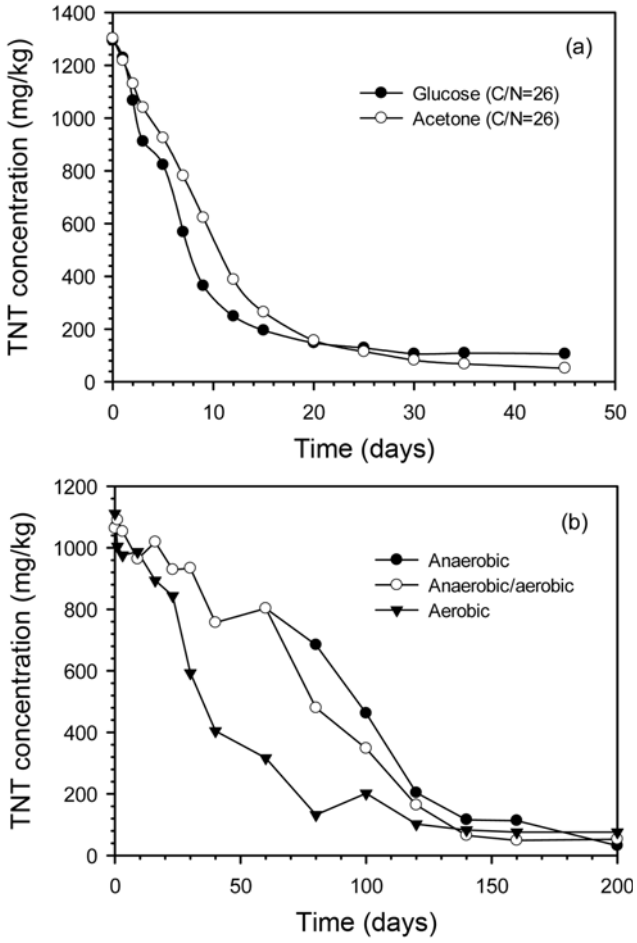

Fig. 1. Time profile of TNT concentration during composting (a) and different metabolic regimes of slurry phase bioreactor (b).

bic treatment was decreased after subsequent aerobic stage. After 200 days of operation, however, degradation efficiency was above 95% in both anaerobic and anaerobic/aerobic treatment. Among the different metabolic regimes studied, the most rapid degradation of TNT was observed in aerobic treatment at the early stage of operation and its degradation efficiency was 71.5% until 60 days [19]. This was about three times larger than that of anaerobic treatment. Craig et al. [23] evaluated slurry phase bioremediation technologies of TNT-contaminated soil. Removal efficiency of anaerobic treatment was 97.1% after operation of 150 days, while aerobic treatment showed 99.1% within 35-70 days.

2. *Salmonella* Microsomal Mutagenecity Assay

The Ames assay is a well-known bacterial mutagenicity test and reverse $His^- \rightarrow His^+$ mutations are visualized by plating *Salmonella typhimurium* bacteria in histidine-poor growth medium. In its classical form the strains TA98 for the detection of frame-shift mutations and TA100 for the detection of base-pair substitution mutations are the most commonly used. Approximately 90% of the chemicals proven to be carcinogens are mutagens; they cause cancer by inducing mutations in somatic cells [24]. Many chemicals are not mutagenic (or carcinogenic) in themselves, but become converted into mutagens (and carcinogens) as they are metabolized by the body. This is the reason the Ames assay includes a mixture of liver enzymes (S9 mix) as metabolic activator.

The number of revertant colonies of *S. typhimurium* in the initial sample before composting is provided in Table 1. The mutagenic activity of the samples was assayed on the basis of a quantitative comparison of the number of revertant colonies (Rt) induced by the test sample and the number induced by negative control (Rc), that is, the number of colonies growing spontaneously. The index of mutagenicity (IM) is defined as IM=Rt/Rc and its two-fold increase was considered a positive result [21,25]. Dimethylsulfoxide (DMSO) was used as negative control in this research. In negative control, the strain TA 98 and TA 100 showed a spontaneous revertant rate per plate of 24-33 and of 38-49. According to the dilution level (×1, ×3, ×9), dose-dependence was shown in both strains of *S. typhimurium* TA98 and TA100. Therefore, all samples were considered a positive result. The mutagenicity in strain TA100 was higher than that in strain TA98. However, both strains of TA98 and TA100 showed relatively low mutagenicity with S9 metabolic activation.

Won et al. [26] reported that TNT was detected as a frame-shift mutagen that significantly accelerates the reversion rate of a frame-shift tester, TA98. In contrast, the major microbial metabolites of TNT appeared to be non-mutagenic. Tan et al. [27] observed that TNT was mutagenic for *S. typhimurium* without the need of a rat

Table 1. Number of revertant colonies of *S. typhimurium* in the initial sample before composting

Sample	Dilution	Number of revertants/g (*S. typhimurium*)			
		TA98		TA100	
		w/ S9 mix*	w/o S9 mix**	w/ S9 mix	w/o S9 mix
Initial sample	×1	10,520±594***	22,850±1103	32,750±2121	37,100±2687
	×3	4,965±64	12,685±516	13,885±191	21,700±424
	×9	1,135±49	3,100±28	2,920±113	6,325±106
Negative control****	DMSO	24±5	33±4	49±1	38±4
Positive control*****	2AA	173±25		242±10	
	4NQO		131±13		1,484±36

*,**Toxicity measured with (+S9) and without (−S9) metabolic activation

***Each value represents the mean±SD (standard deviation)

****Dimethylsulfoxide (DMSO) was used as negative control for the corresponding strains

*****2-Aminoanthracene (2AA) and 4-nitroquinoline-1-oxide (4NQO) was used as positive control for the corresponding strains

Table 2. Number of revertant colonies of *S. typhimurium* strains TA98 and TA100 before and after composting

Sample	Dilution	Number of revertants/g (*S. typhimurium*)			
		TA98		TA100	
		w/ S9 mix*	w/o S9 mix**	w/ S9 mix	w/o S9 mix
Composting	×1	10,520±594***	22,850±1103	32,750±2121	37,100±2687
(initial sample)	×3	4,965±64	12,685±516	13,885±191	21,700±424
	×9	1,135±49	3,100±28	2,920±113	6,325±106
Glucose	×1	1,018±8	1,140±85	1,082±19	1,339±1
	×3	240±25	456±34	802±3	270±11
	×9	67±0	114±8	127±4	76±8
Acetone	×1	659±12	2,369±58	832±68	971±49
	×3	625±24	1,183±26	202±3	381±89
	×9	42±2	90±4	63±4	54±5
Slurry phase	×1	10,475±658	24,185±3001	33,893±1911	38,760±1895
(initial sample)	×3	3,727±319	8,665±764	11,632±2022	3,649±578
	×9	914±5	1,256±61	2,652±215	717±66
Anaerobic	×1	520±16	2,436±22	313±18	882±87
	×3	306±8	964±6	241±1	462±25
	×9	108±4	561±1	187±4	169±1
Anaerobic	×1	394±8	936±8	288±17	256±13
/aerobic	×3	208±11	215±12	178±8	98±3
	×9	47±4	51±3	112±12	49±1
Aerobic	×1	357±10	1,242±280	889±12	979±41
	×3	185±14	354±8	681±16	261±44
	×9	50±2	89±2	136±6	117±4

*,**Toxicity measured with (+S9) and without (−S9) metabolic activation
***Each value represents the mean±SD (standard deviation)

liver metabolic activation system (−S9). The mutagenic potency of TNT decreased in proportion to the number of nitro groups that were reduced to the amino form. The presence of a nitro group on the 4 position of the diamino congener is necessary for mutagenicity. Among the active congeners, mutagenicity was generally greater for TA100 than TA98, except that for the 4-amino congener the reverse was true. Padda et al. [28] also reported that TNT and all its metabolites were mutagenic in either strain TA98 or TA100 without S9 metabolic activation.

Table 2 shows the number of revertant colonies of *S. typhimurium* strains TA98 and TA100 before and after bioremediation processes for composting and slurry phase bioreactor processes. According to the dilution level for composting, dose-dependence was shown in all strains (TA98 and TA100) and metabolic activation (with S9 mix and without S9 mix). In strain TA98 with S9 metabolic activation, the mutagenicity after composting was decreased from 10,520 rev/g (initial sample) to 1,018 rev/g (glucose addition) and 659 rev/g (acetone addition) (Table 2 and Fig. 2). In strain TA100 with S9 metabolic activation, the mutagenicity after composting was decreased from 32,750 rev/g (initial sample) to 1,082 rev/g (glucose addition) and 832 rev/g (acetone addition). Higher mutagenicity was observed without S9 metabolic activation compared with S9 metabolic activation in all samples including before and after composting. Tan et al. [27] observed that when S9 was included in the Ames assay, there was always a decrease in the number of revertants induced as compared with those without S9 metabolic activation. The mutagenicity in strain TA100 was higher compared to strain TA98. Honeycutt et al. [29] also reported that TNT was mutagenic in strain TA98 both with and without S9 metabolic activation, and the mutagenicity was low in case of with S9 metabolic activation.

The number of revertant colonies of *S. typhimurium* strains TA98 and TA100 before and after slurry phase bioreactor operation under different metabolic regimes is also shown in Table 2 and Fig. 3. The dose-dependent displayed similar trends in all strains (TA98 and TA100) and metabolic activation (with S9 mix and without S9 mix) according to the dilution level as described in composting. Higher mutagenicity was observed without S9 metabolic activation compared with S9 metabolic activation in all samples, except for the case of anaerobic/aerobic treatment on strain TA100. In strain TA98 with S9 metabolic activation, the mutagenicity after slurry phase bioreactor operation was decreased from 10,475 rev/g (initial sample) to 520 rev/g (anaerobic treatment), 394 rev/g (anaerobic/aerobic treatment), and 357 rev/g (aerobic treatment), respectively. In strain TA100 with S9 metabolic activation, the mutagenicity after slurry phase bioreactor operation for 200 days was decreased from 33,893 rev/g (initial sample) to 313 rev/g (anaerobic treatment), 288 rev/g (anaerobic/aerobic treatment), and 889 rev/g (anaerobic treatment), respectively.

3. Toxicological Comparison between Two Processes

Table 3 and Fig. 4 show the toxicological reduction percent of residuals after composting and slurry phase bioreactor operation. In strain TA98 with S9 metabolic activation, the percentage reduction

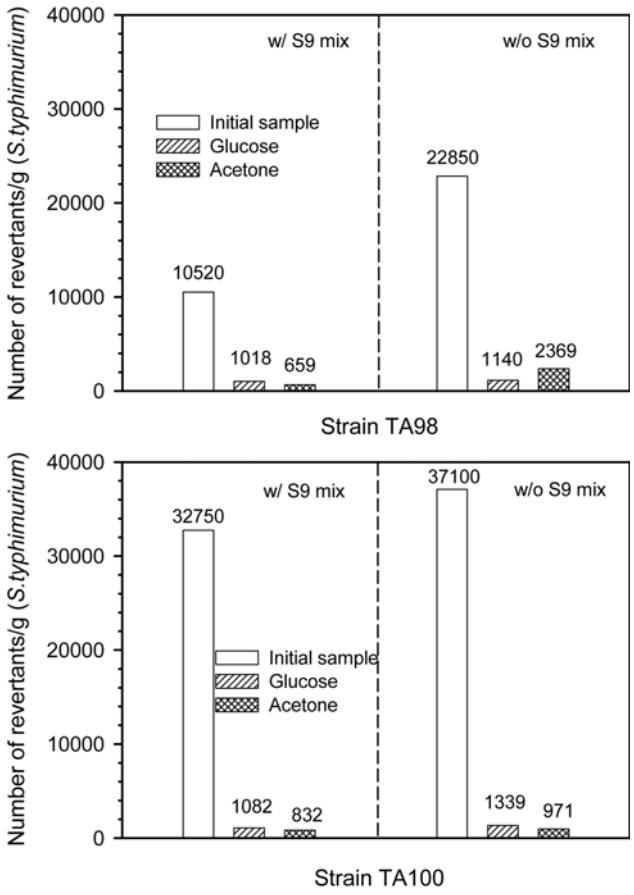

Fig. 2. Comparison of mutagenicity on *S. typhimurium* strains TA98 and TA100 before and after composting (Data show the number of revertant colonies of *S. typhimurium* strains TA98 and TA100, w/ and w/o S9 mix).

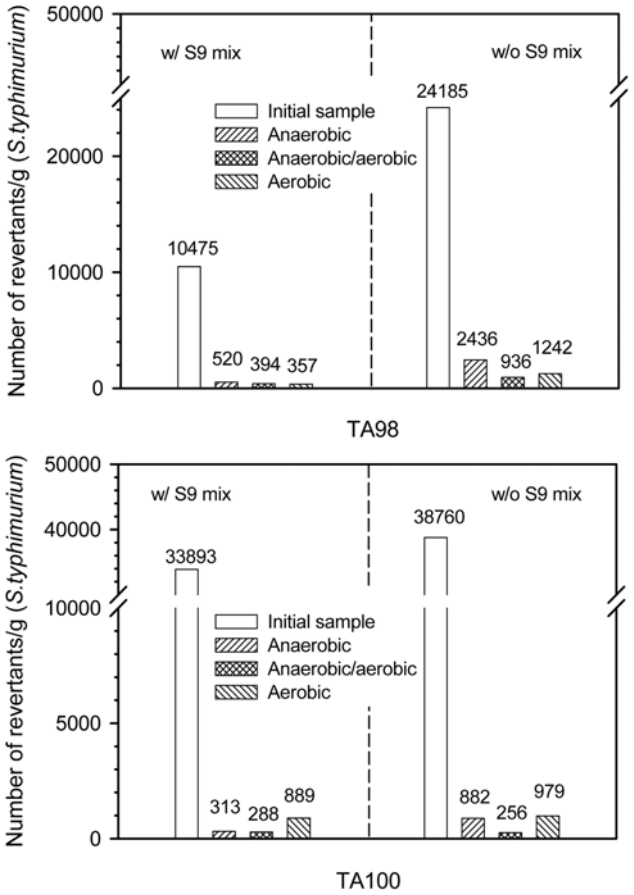

Fig. 3. Comparison of mutagenicity on *S. typhimurium* strains TA98 and TA100 before and after slurry phase bioreactor operation under different metabolic regimes (Data show the number of revertant colonies of *S. typhimurium* strains TA98 and TA100, w/ and w/o S9 mix).

of final composts was 90.3% for glucose addition and 95.0% for acetone addition, respectively. In strain TA100 with S9 metabolic activation, the percentage reduction of final composts was 96.7% for glucose addition and 97.5% for acetone addition. More reduction was observed in strain TA100 with S9 metabolic activation. These results were similar to those of previous study by Griest et al. [30], who performed composting of TNT-contaminated soil and evaluated for mutagenicity of the compost materials. As a result, the mutagenicity of organic solvent extracts from the compost materials decreased more than 90% in some cases after composting.

In strain TA98 with S9 metabolic activation, the toxicological percentage reduction of final residuals was 95.0% for anaerobic treatment, 96.2% for anaerobic/aerobic treatment, and 96.6% for anaerobic treatment, respectively (Table 3 and Fig. 4). In strain TA100 with S9 metabolic activation, the percentage reduction of final residuals was 99.1% for anaerobic treatment, 99.2% for anaerobic/aerobic treatment, and 97.4% for anaerobic treatment. More toxicological reduction was observed in strain TA100 with S9 metabolic activation as described in composting. Rocheleau et al. [31] evaluated the

Table 3. Comparison of the reduction percent of residuals after composting and slurry phase bioreactor operation

Sample	Conditions	Mutagenicity			
		TA98		TA100	
		With S9 mix*	Without S9 mix**	With S9 mix	Without S9 mix
Composting	Glucose	90.3	95.0	96.7	96.4
	Acetone	93.7	89.6	97.5	97.4
Slurry phase bioreactor	Anaerobic	95.0	89.9	99.1	97.7
	Anaerobic/aerobic	96.2	96.1	99.2	99.3
	Aerobic	96.6	94.9	97.4	97.5

*,** Toxicity measured with (+S9) and without (−S9) metabolic activation

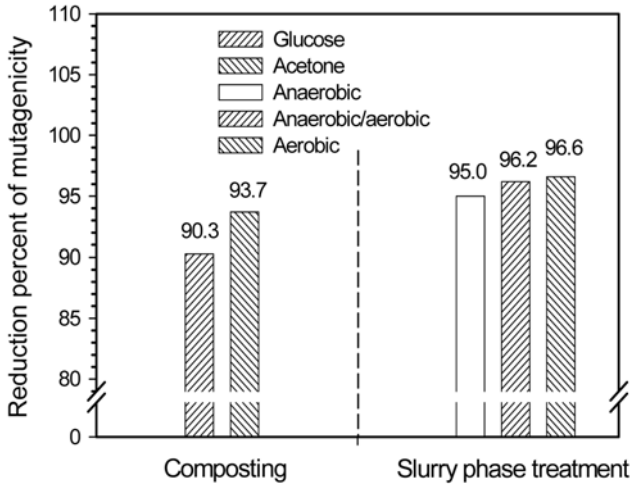

Fig. 4. Comparison of mutagenicity reduction after composting and slurry phase treatment (Reduction percent is calculated based on the number of revertants on *S. typhimurium* strain TA98 with metabolic activation).

toxicological effects of slurry phase bioreactor treating TNT-spiked soil with various toxicity assays such as microtox, green algea growth inhibition, bacterial genotoxicity and mutagenicity, and earthworm mortality and growth inhibition. At the end of the slurry phase treatment, data showed reduction of all bioassays, including mutagenicity by the Ames assay.

Likewise, the reduction of toxicity, much more reduction of mutagenicity was observed in slurry phase treatments than composting. In comparison of toxicity reduction between two processes, slurry phase treatment was a more effective process than composting.

CONCLUSIONS

The number of revertant colonies of all samples, including composting and slurry phase bioreactor, was more than two-fold compared with that of negative control. Also, dose-dependence was shown in both strains of *S. typhimurium* TA98 and TA100, according to the dilution level. Therefore, all samples were considered a positive result. In case of the compost of the contaminated soil, greater reduction of mutagenicity was observed in acetone addition experiment except the case of strain of *S. typhimurium* TA98 without S9 metabolic activation. There was no significant difference of mutagenicity reduction among the three slurry phase bioreactors.

In both cases of composting and slurry phase bioreactor processes, mutagenicity reduction was found in strain TA100 much more than strain TA98. Compared with mutagenicity reduction between composting and slurry phase bioreactor, composting was lower than slurry phase treatment. This indicated that slurry phase treatment was a more effective process than composting in toxicological reduction. This research has the advantage of speed and ease of performance in comparison to testing of other higher life forms due to the shorter life cycles of microorganisms.

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