# CONTRIBUTION OF BACTERIA ON THE INSOLUBLE TECHNETIUM FORMATION IN PADDY SOIL SURFACE WATER

N. Ishii, K. Tagami, S. Uchida
Office of Biospheric Assessment for Waste Disposal, National Institute of Radiological Sciences,
4-9-1 Anagawa, Inage-ku, Chiba-shi, 263-855 Japan

## ABSTRACT

To understand the formation mechanisms of insoluble technetium (Tc), we studied microorganisms causing the Tc insolubilization in surface water covering paddy fields. Five types of paddy soils were flooded and incubated for 7 days. After the incubation, the surface water covering soils were used for the following radioactive tracer experiments: The surface water samples were cultured for 4 days with $^{95m}TcO_4^-$, and the insoluble Tc was separated from the samples by a 0.2 µm-pore-size membrane filter. Among the surface water samples, relative high amounts of insoluble Tc (>70% of total $^{95m}Tc$) were found in gley and gray lowland soil samples. Both soil types were commonly used for rice cultivation in Japan. Thus, Tc may be easy to accumulate on paddy field soils in Japan. To clarify the kind of microorganisms contributed on the formation of insoluble Tc, the gray lowland soil samples was pretreated to make two different surface water samples: 1) a sample inhibited fungal activity and 2) a sample inhibited bacterial activity. The addition of fungicide to the gray lowland soil sample caused the increase of insoluble Tc amount. In contrast, the addition of antibiotics on bacteria resulted in the decrease of insoluble Tc amount. These results showed that the formation of insoluble Tc caused by bacteria.

## INTRODUCTION

Technetium-99 ($^{99}T$) is a radioactive contaminant in marine (1) and terrestrial environments (2). The sources of $^{99}Tc$ in the environment were primarily due to fallout from nuclear weapons testing, nuclear fuel reprocessing, and the disposal of low- and intermediate-level wastes. The half-life of $^{99}Tc$ is $2.1 \times 10^5$ years, and thus this radionuclide will persist in the environment for many years. For these reasons, it is necessary to clarify the behavior of $^{99}Tc$ in the environment.

The behavior of $^{99}Tc$ depends on its chemical form. The most stable chemical species of Tc under aerobic conditions is $TcO_4^-$ (3). This chemical form is both highly soluble and mobile in the environment (4). In addition, the availability of $TcO_4^-$ to plants is high (5), suggesting the concern of the transfer of this radioactive nuclide from plants to human beings. In contrast to $TcO_4^-$, several low-valence oxides of Tc such as $TcO_2$ and $TcO(OH)_2$ are both insoluble and immobile due to strong sorption of this species by solid materials (3). Insoluble form of Tc was often observed under anaerobic conditions. The low valence Tc (IV) could be unavailable for agricultural plants (6, 7).

In paddy fields, oxygen in plow layer is consumed by microbial decomposition of organic matter during the irrigation period of paddy. Consequently, the plow layer becomes anaerobic conditions involving a low redox potential (*Eh*). These conditions in the plow layer could cause the accumulation of Tc in soils of paddy fields by the transformation of soluble $TcO_4^-$ to insoluble Tc (IV) (8). Although insoluble Tc formations in waterlogged paddy fields have been studied, little is known about the physicochemical forms of Tc in surface waters covering paddy fields.

The surface water is a habitat for various microorganisms such as virus, bacteria, fungi, protozoa, and algae. Microorganisms have ability to interact with metals, for example, by through their respiration and metabolism. In case of Tc, $TcO_4^-$ is enzymatically reduced to insoluble Tc (IV) by sulfate-reducing bacteria, iron-reducing bacteria, and the enteric bacterium *Escherichia coli* (9). The reduction of Tc by the several experimental bacteria raises the possibility of insoluble Tc formation in the surface water.

Objects of this study, therefore, were to demonstrate the insoluble Tc formation in surface water covering paddy fields and to determine the kinds of microorganisms contribute on the insoluble Tc formation. For these objects, a series of radioactive tracer experiments was carried out using $^{95m}TcO_4^-$.

## MATERIALS AMD METHODS

### Surface water samples

Five types of paddy soils were used in this study (Table I). The soils were air-dried and sieved through a 2 mm mesh. The soil samples, 5 g each, were flooded with 7.5 mL of 0.3% glucose water. The flooded samples were incubated for 7 days at 25˚C in 12-h light-dark conditions. From the samples, 1.8 mL of the surface water covering paddy soils were collected in a new tube for the following radioactive tracer experiment.

Table I. Soil types

| Sample code | Soil type |
|---|---|
| A32 | **A**ndosol |
| B56 | **B**rown |
| G20 | **G**ley |
| L38 | Gray **L**owland |
| Y19 | **Y**ellow |

### Microbial abundance

For enumeration of microorganism cells, the surface water samples were stained with SYBR Gold (Molecular Probes, Inc.), which is a nucleic acid fluorescence dye, and filtered through a 0.2-µm black polycarbonate filter immediately after the collection of the water samples from the flooded cultures. The SYBR-positive cells were counted under epifluorescence microscopy (× 1,000) using blue excitation.

### Formation of insoluble Tc in the surface water samples

Radioactive tracer, carrier free $^{95m}TcO_4^-$, was prepared in deionized water by the method of Sekine et. al. (10). Then the solution of the tracer was sterilized by a filtration passing through a 0.2 µm-pore-size filter. The $^{95m}TcO_4^-$ was added to the 5 kinds of the surface water samples. After the addition of the $^{95m}TcO_4^-$, the samples were statically cultured at 25˚C for 4 days in the dark. The cultured samples were passed through a 0.2-µm pore-size cellulose acetate membrane filter to separate insoluble Tc from the cultures. That is, the size of insoluble Tc was physically more than 0.2 µm. Radioactivity of the $^{95m}Tc$ in the filtrate obtained by the filtration (dissolved fraction) was measured with a NaI(Tl) scintillation counter. Relative amount of insoluble Tc was given by:

$$RA(\%) = \frac{T - D}{T} \times 100$$

where RA = relative amount of insoluble Tc; T = total $^{95m}Tc$ activity; and D = $^{95m}Tc$ activity in dissolved fraction.

### Microbial effects on insoluble Tc formation in the L38 sample

To determine the kinds of microorganisms contribute on the insoluble Tc formation, two subsamples were prepared using the L38 surface water sample. The subsamples were as follows: 1) fungicide sample, and 2) bactericide sample. The fungicide sample was prepared by the addition of cycloheximide to the surface water sample at a final concentration of 50 µg mL$^{-1}$ to inhibit the fungal growth. The bactericide consisting of ampicillin (Ap), streptomycin (Sm), and tetracycline (Tc) was used to inhibit bacterial growth. Each component of the bactericide was added to the surface water sample at a final concentration of 25 µg mL$^{-1}$ each. The addition of these reagents to the L38 surface water sample was followed by the addition of $^{95m}Tc$. The subsequent procedure of this radioactive tracer experiment was the same as that for "formation of insoluble Tc in the surface water samples" mentioned above.

The redox conditions of the L38 surface water were determined using a redox indicator, resazurin. The reagent was added to the surface water immediately after the sample collection from the flooded paddy soil at the final concentration of 2 µg mL$^{-1}$. The color of resazurin in the surface water was observed at day 0 and 4 of the addition.

## RESULTS AND DISCUSSION

### Insoluble Tc formation in surface water covering paddy soils

The formation of insoluble Tc in the surface water samples was determined in the radioactive $^{95m}$Tc tracer experiment using the surface water samples of the 5 kinds of paddy soils (Fig. 1). The amounts of insoluble Tc differed among the samples. The relatively high amounts of insoluble Tc were found in G20 and L38 samples and the amounts were more than 70% of the total added Tc. This result suggests that insoluble Tc could form in surface water covering paddy fields. For the other samples, the insoluble Tc amounts were less than 35% of total. Therefore, it seemed that insoluble Tc was not always formed in surface water of waterlogged paddy fields but formed in surface water under specific conditions. The conditions may relate to the water quality such as pH and *Eh*. The different values of pH and *Eh* in surface waters were depended on soil types (11). Thus, soil types would be one of the important parameters for the insoluble Tc formation in surface water. The surface water samples of G20 and L38 were gley and the gray lowland soil, respectively. Both soil types were commonly used for rice cultivation in Japan. Thus, Tc could be easy to accumulate on paddy field soils in Japan.

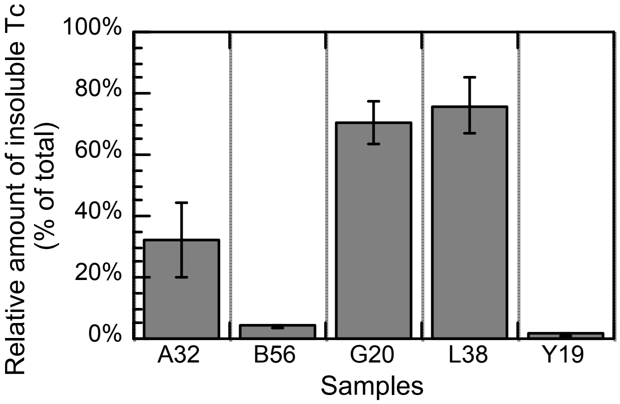

Fig. 1 Relative amounts of insoluble Tc in surface water samples. Columns and error bars indicate the mean and standard deviation of three replicates, respectively.

In the surface water samples, most of the SYBR-positive particles were microbial cells, which were characterized as spheres and rods (Fig. 2). Little inorganic particles, which were able to discriminate from microbial cells by their figure and fluorescence color, were observed. Further, the number of protozoa and algae was also negligible. The microscopic observations indicated that the microbial composition of the surface water samples were mainly bacteria and fungi.

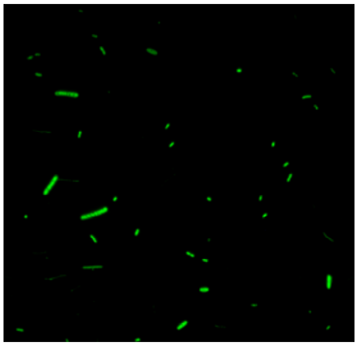

Fig. 2 Photograph of SYBER-Gold positive particles in the surface water of L38. Little protists and inorganic substances were observed.

The microbial abundances in the surface water samples were determined by epifluorescence microscopy (Fig. 3). The number of microbial cells differed among the samples. The highest abundance was 6.2 × $10^7$ cells $mL^{-1}$ of L38, followed in order by G20, B56, A32 and Y19. This abundance order was similar to that of the insoluble Tc amount, therefore, a part, but not all, of insoluble Tc formation may be explained by the microbial abundance. The low amount of insoluble Tc in the B56 surface water sample, which could not be explained by the microbial abundance, indicated that the insoluble Tc formation was not controlled only by the microbial abundance. In addition to the abundance, microbial species may also affect on the insoluble Tc formation. The formation of insoluble Tc in surface water samples would be caused by microbial cells at least. This hypothesis was also supported by our previous study indicated that a negligible amount of insoluble Tc was formed in a surface water sample with no living microorganisms (11).

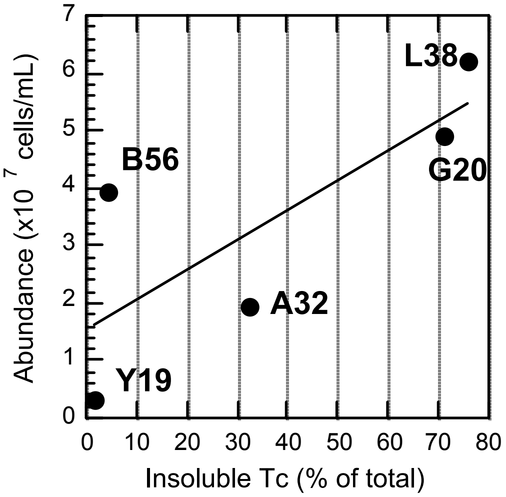

Fig. 3 Relationship between microbial abundance and insoluble Tc amount in surface water samples.

### Kind of microorganisms contributed on the insoluble Tc formation

The relative amounts of insoluble Tc in the L38 surface water sample exposed to three treatments to assess the microbial effect are given in Table II. In this experiment, the samples were cultured in the dark. In addition, little algae and protozoa were observed in the L38 sample (Fig. 2). This observation suggested that the contribution of these protists on the in soluble Tc formation was negligible compared to that of bacteria and fungi.

The formation of insoluble Tc by bacteria and fungi were determined using two kinds of subsamples (Table II). The addition of fungicide to the L38 surface water sample showed a little increase in the insoluble Tc amount, but the difference in the amounts between fungicide and untreated samples was not significant. In contrast to the result of the fungicide experiment, the addition of bactericide to the L38 sample resulted in the remarkable decrease in the relative amount of insoluble Tc. The difference in the relative amounts of insoluble Tc between the untreated and bactericide samples was obviously significant (student's *t*-test, $P < 0.01$). These results showed that most of the insoluble Tc formation in the surface water was caused by bacteria, rather than by fungi.

Table II. Relaive amount of insolble Tc in L38 and its subsamples

| Treatment | Subsample name | Insoluble Tc (% of total) mean ± sd | n | P value |
|---|---|---|---|---|
| 0 | L38 surface water (untreated) | 70.7 ± 0.4 | 3 | |
| 1 | fungicide | 77.8 ± 9.2 | 3 | > 0.05 |
| 2 | bactericide | 19.7 ± 1.6 | 3 | < 0.01 |

Sulfate-reducing bacteria (SRB), which live even in paddy fields, are able to reduce and precipitate $TcO_4^-$ enzymatically (12). Since the formation of insoluble Tc by the SRB was thought, the suitability of the surface water condition for the survival of obligate anaerobes SRB was examined using a redox indicator, resazurin (Fig. 4). Generally, SRB require a complete absence of molecular oxygen and a highly reduced environment to function efficiently. Under these anaerobic and reduced conditions, resazurin becomes color-less. The color of the surface water, however, changed to pale brown to pink. This color change indicated that the surface water was under a reductive condition with not a complete lack of oxygen. The pinkish color did not change during the incubation of the sample. No color change of the sample suggested SRB did not effectively work for the insoluble Tc formation in the surface water. Therefore, the insoluble Tc could be formed by iron-reducing bacteria and another bacteria other than SRB.

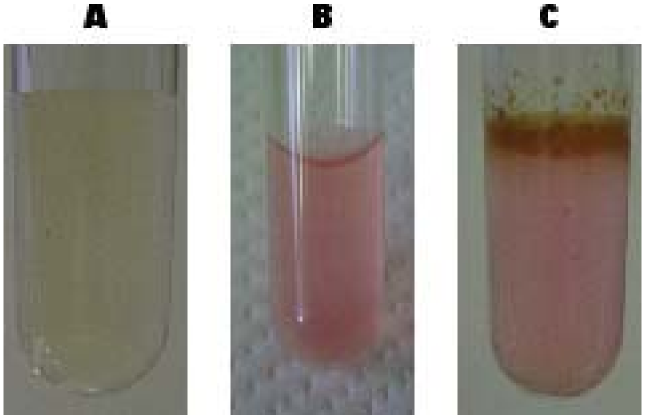

Fig. 4 Photographs of the L38 surface water sample. A) immediately after the collection of the surface water, B) immediately after the addition of resazurin, and C) 4 days from the resazurin addition.

After the incubation of the surface water sample for 4 days, an iron-oxide-like-film was observed on the top of the sample (Fig. 4 C). The development of the film could result from the oxygen supply from a headspace of the sample because the sample was incubated statically. Consequently, oxygen dissolved into the sample solution, and the provision of oxygen would inhibit SRB activity.

Another characteristic of the bacteria in the surface water was "dry-tolerant", because the air-dried soils were used in this experiment.

## CONCLUSION

The results provided the evidence to support the formation of insoluble Tc in the surface water covering paddy fields. The insoluble Tc was especially formed in the surface water covering Japanese typical paddy soils, both gley and gray lowland soils. The formation of insoluble Tc in the surface water would also be one of Tc accumulation mechanisms in paddy fields.

The bacterial contribution on the insoluble Tc formation was suggested by the inhibition of its formation by the addition of bactericide to the surface water. Although it has been known that SRB reduce and precipitate $TcO_4^-$, bacteria causing insoluble Tc in the surface water would not be SRB. The surface water was cultured statically in this experiment, and consequently the conditions of the surface water were reductive but not complete lack of oxygen.

From this series of radioactive experiments, it showed the possibility that insoluble Tc was formed by bacteria living in the surface water covering Japanese paddy fields. In the future, isolation and identification of Tc insolubilizing bacteria are required to clarify the behavior of Tc in paddy fields.

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