

Safety of Oppenheimer's microbial products



Oppenheimer
Biotechnology Inc.

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Pages 3-5 Confirmation test of pathogenic and toxic production on degradation process [Microorganisms in the Oppenheimer Formula are not pathogenic nor do they generate toxic by-products]

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[Microbes will die after consuming their food [oil].]

1. Pathogen test

1) Check of presence of pathogen

Samples of our microbial products were sent to the Texas State Health Department for analysis to determine whether if pathogen presents or not. The results are shown in the table below;

Analytes	Detection
<i>Salmonella</i> bacteria	None
<i>Shigella</i> bacteria	None
<i>Campylobacter</i> bacteria	None
<i>Yersinia</i> bacteria	None
<i>Vibrio cholerae</i>	None
<i>Vibrio parahaemolyticus</i> and related to <i>Halophi Vibrios</i> MPN	<?3/g
<i>Staphylococcus aureus</i> MPN	<?3/g

Pathogens analysis results report,

2) Check of presence of pathogen by Aliphatic acid identification technique.

Samples of our microbial products were sent to ACCU Lab Newark, Delaware, USA for the analysis by using Aliphatic acid identification technique to determine whether if pathogen presents or not. No matches were identified in the sample against the known pathogens listed in the database. The table below provides names of major 12 pathogens for this analysis and all the analysis results demonstrated as 'Not detected'.

<i>Aeromonas spp.</i>	<i>Bacillus megaterium</i>	<i>Camylobacter spp.</i>
<i>Clostridium perfringens</i>	<i>Clostridium botulinum</i>	<i>E. coli</i>
<i>Legionella spp.</i>	<i>Pseudomonas marginalis</i>	<i>Salmonella spp.</i>
<i>Shigella spp.</i>	<i>Staphylococcus aures</i>	<i>Yersinia enterocolitica</i>

Pathogen analysis results report (Aliphatic acid identification technique),

3) Check of presence of pathogen by Aliphatic acid identification technique

Samples of our microbial products were sent to ACCU Lab Newark, Delaware, USA for the analysis by using Aliphatic acid identification technique to determine whether if pathogen presents or not. No matches were identified in the sample against the known pathogens listed in the database. The table below provides names of major 19 pathogens for this analysis and all the analysis results demonstrated as 'Not detected'.

<i>Aeromonas sp.</i>	<i>Aspergillus fumigatus</i>	<i>Bacillus megaterium</i>
<i>Bacillus anthracis</i>	<i>Camylobacter sp.</i>	<i>Clostridium botulinum</i>
<i>Clostridium perfringens</i>	<i>Candida albicans</i>	<i>E. coli</i>
<i>Enterococcus sp.</i>	<i>Fecal coliform bacteria</i>	<i>Legionella sp.</i>
<i>Listeria monocytogenes</i>	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas marginalis</i>
<i>Salmonella sp.</i>	<i>Staphylococcus aures</i>	<i>Vibrio sp.</i>
<i>Yersinia enterocolitica</i>		

Pathogen analysis results report (Aliphatic acid identification technique),

Analysis results report (translation)

Certificate of Analysis

No.02-04169

H14-C301-210

Client: BioRangers Inc.

Yagai Science Inc.

Naho-cho 12-2-39,

Higashiku, Sapporo City, JAPAN

TEL (011)751-5151

Registered laboratory; Drinking water analysis

(Hokkaido governor registration: Hokkaido 56 Water 4),

Analysis certification entity (Hokkaido governor registration: No. 607)

Contact person; Miho Shikata

We certify the sample analysis results as below;

Analysis description : Pathogen test

Samples : Formula, TerraZyme

Analysis report

Analysis description	Unit	Oppenheimer Formula solution	TerraZyme solution
	MPN/100ml /ml	ND 9	ND 5

Sample solution: 1g from each sample was diluted with 10ml of normal saline solution (sterilized).

Analysis method: E.coli, Environmental Agency Dec. Table No. 59,

General pathogens, Drinking water analysis method, ver. 2001

MICROTOX™ TEST

In order to determine whether Oppenheimer's formula generates hazardous compounds such as surfactant substances and has hazardous impacts on other lives, confirmative tests by using MICROTOX TEST™ were undertaken. This is an analysis method which provides determination whether if unknown hazardous chemical substance presents in the sample or not, which results are commonly accepted in US. The scheme of this method is to determine whether microorganisms in a sample which are cultivated in a solution with luminous bacteria have toxicity against these bacteria by measuring their luminescence intensity (disappearance of luminescence). By using the obtained value as an indicator, a judgment whether additive substances have toxicity onto lives or not can be made. A confirmative test using this technique for the Oppenheimer Formula was undertaken conforming to the method of Canadian EPA. The samples were sent for the analysis to GRACE Dearborn Inc in Mississauga City, Ontario, Canada. The results are shown in the table below;

Description	Result
Concentration of subject microbial products (Oppenheimer's formula)	10,000mg/L
Sample number	3
% Light loss 5 minutes 15 minutes 30 minutes	

MICROTOX™ TEST REPORT

In MICROTOX™ TEST, determination of toxicity is made by observing disappearance of luminescence from the bacteria in following designated exposure time - respectively 5, 15 and 30 minutes. As the results in the table above, no significant difference as of Light loss between all exposure time (5, 15 and 30 minutes) was observed. Therefore subject microorganisms were determined to not have toxicity onto other living organisms.

2. Impact on aquatic animals

[No adverse impact on the ecosystems was identified.]

Evaluation of effects on freshwater and saltwater fishes

Blue-green pullers

A short time breeding test (7 days, acute toxicity test) using blue-green pullers was undertaken. Since no demise of the blue-green pullers by addition of Oppenheimer's formula into their living water was observed, the formula was determined to have no acute toxicity.

Sweetfishes, *Plecoglossus altivelis*

A short time breeding test (7 days, acute toxicity test) using sweet fishes was conducted. Since no demise of the fishes in the water tank where Oppenheimer's formula was added was observed, the formula was determined to have no acute toxicity. A long time breeding test (30 days, subacute toxicity test) was also undertaken. Some demises from both experimental groups (where the formula was added and not added), however, no significant difference nor abnormality was identified in anatomies for the both groups. No subacute toxicity in the formula is therefore considered to be present.

Rainbow trout

A short time breeding test (4 days, acute toxicity test) was undertaken to examine toxicity of breeding water samples at various concentrations of Oppenheimer's formula. No toxicity of the formula was identified.

Silversides, *amenidia beryllina*

A short time breeding test was conducted using actual treated water from a real contaminated site where bioremediation was applied. No toxicity of the microorganisms onto Silversides' lives was observed in water samples at various concentrations of microbial products during 96 hours breeding period.

Acute and Subacute Toxicity Tests

Short and long time breeding tests (acute and subacute toxicity tests) were respectively undertaken to evaluate safety of the Oppenheimer Formula onto aquatic lives.

Effects on lives of Blue–green pullers

(a short time breeding test in 7 days acute toxicity test .

10 Blue-green pullers (average length: c.a. 3 cm, average weight: c.a. 1 g from each experimental group were observed in 20 L of artificial seawater under condition of 28 temperature and feeding 3 times per day morning, noon and evening for a 7 days period. The Oppenheimer Formula was added into the seawater at 0.1% concentration for one culture (a test group), and the other culture was fed under the formula free condition (a control group). (A microbial products addition test).

Survival rates of Blue-green pullers short time breeding test in 7 days

	Number of demise	Number of survival	Survival rate %
Control group	0	10	100
Test group	0	10	100

Given the results above, no acute toxicity of the formula onto Blue-green pullers was identified.

Effects on lives of Sweetfishes, *Plecoglossus altivelis* fries

(a short time breeding test in 7 days acute toxicity test

20 Sweetfish fries average length: c.a. 8cm, average weight: c.a. 5g from each experimental group were observed in 100 L of freshwater (groundwater) under condition of 19 20 temperature and feeding 3 times per day morning, noon and evening for a 7 days period. The Oppenheimer Formula was added into the freshwater at 0.1% concentration for one culture (a test group), and the other culture was fed under the formula free condition (a control group). (A microbial products addition test).

Survival rates of Sweetfish fries short time breeding test in 7 days

	Number of demise	Number of survival	Survival rate %
Control group	0	20	100
Test group	0	20	100

Given the results above, no acute toxicity of the formula onto Sweetfish fries was identified.

3 Effects on lives of Sweetfishes, *Plecoglossus altivelis* fries

(a long term breeding test in 30 days subacute toxicity test

20 Sweetfish fries average length: c.a. 8cm, average weight: c.a. 5g from each experimental group were observed in 80 L of freshwater (groundwater) under condition of 19 20 temperature and feeding 3 times per day morning, noon and evening for a 30 days period. The Oppenheimer Formula was added into the freshwater at 0.1% concentration for one culture (a test group). (A microbial products addition test).

Survival rates of Sweetfish fries long time breeding test in 30 days

	Number of demise	Number of survival	Survival rate %
Control group	5	15	75
Test group	4	16	80

Several demises of Sweetfish fries were observed both in the Control group and the Test group, however latter numbers were slightly less the former. Anatomical results of the dead fries showed no abnormality on these fries. Given that the demises were intensively observed around one week after the start of the test, it is assumed that the demises were caused by the shock due to changes of their living environment. Therefore, no subacute toxicity against Sweetfish fries is considered to be present in the formula.

Reference Bioremediation on the shore after an oil spill from the Nakhodka in the Sea of Japan, II. Toxicity of a bioremediation agent with microbiological cultures in aquatic organisms, Marine Pollution Bulletin 40 308-324 (2000)

Effects on lives of rainbow trout

(a short time breeding test in 4 days acute toxicity test

Rainbow trout average length: c.a. 30mm, average weight: c.a. 0.25g from each experimental group in 10 L of dechlorinated tap water with 15 °C temperature were observed for a 4 days period. The Oppenheimer Formula was added into the tapwater for the Test group at 0.1% concentration (10,000mg/L). (A microbial products addition test).

In the results, the LC₅₀ of microorganisms in the formula was obtained as of >10,000 mg/L. Given this value, no acute toxicity onto Rainbow trout is considered to exist in the formula.

Note: LC₅₀ ; Median Lethal Concentration,

The concentration level of a test solution that is assumed to have lead 50% of experimental lives' demises. This value is used as an indicator which shows levels of acute toxicity on aquatic lives, which of the lower means the higher toxicity.

Example LC₅₀ against Rainbow trout of surfactant LAS, linear alkylbenzene sulfonate Surfactant which is normally used 4.7mg/L)

Reference Report, BEAK CONSULTANTS LIMITED (Canada)

5 Acute toxicity test on Silversides, *amenidia beryllina*

The Oppenheimer Formula was applied to the bioremediation for oil spill from the Mega Borg in the Gulf of Mexico. A series of acute toxicity tests using Silversides, *amenidia beryllina* were undertaken by using the actual water samples from this remediation site.

A course of breeding test using Silversides was undertaken by adding the formula at various concentration in three sets of experimental water samples with silversides; a control, a non treated and a treated samples.

For the result, after 96 hours breeding tests, no LC₅₀ of Silversides was recorded in any experimental sample sets or at any concentrations of microbial products. A few demise of Silversides were observed during the experiments, however, no correlation between these demises and concentrations of added microbial products was identified. Given that the growth condition of the silversides in the control sample was observed to be fine, these fishes used for this breeding test were considered to be suitable for the series of experiments.

These above results demonstrate that no toxicity of Oppenheimer's formula onto Silversides was identified.

Reference Environmental Research Institute, US EPA,

Evaluation of effects on freshwater and saltwater invertebrates

Water flea, *ceriodaphnia dubia*

Short time breeding tests (acute toxicity test, 2 days) were conducted to determine impacts of Oppenheimer's formula on water flea cultures at various concentrations of the formula. No acute toxicity was identified.

Mysid shrimps, *Misidopsis bahia*

Using real treated water samples from a bioremediation site, effects of Oppenheimer's formula on living organisms were evaluated. Short breeding tests (in a 96 hours period) of Mysid shrimps at various concentration of the formula demonstrated that no toxicity of the formula to be present.

Rotifers

A series of bioassays was conducted under two conditions - restricting the presence of microbial contaminants and not restricting the presence of microbial contaminants. - to compare rotifer culture growth. Above both two groups were prepared respectively as Oppenheimer's formula added and non-added. Since no significant difference between samples were observed, no effect of the formula is considered to be present.

Echinus, *Toxopneusts pileolus*

Fertilization tests were undertaken by comparing effects of Oppenheimer's formula, oil dispersant and household neutral detergent on eggs of Echinus. The results show that effects of the formula were demonstrated to be much less than others.

Acute toxicity tests on aquatic lives

1. Effects on lives of Water flea, *ceriodaphnia dubia*

(a short time breeding test in 2 days acute toxicity test

Water flea in 200 ml of dechlorinated tap water with the temperature of 20 was observed for a 2 days period. The Oppenheimer Formula was added into the tapwater for the Test group at 0.1% concentration (10,000mg/L). (A microbial products addition test).

For the results of the breeding test, a value of LC₅₀* for the microorganisms was obtained as of >10,000 mg/L. Given this value, no acute toxicity of the formula onto Water flea is considered to be present.

Laboratory BEAK CONSULTANTS LIMITED

2) Acute toxicity test on Mysid shrimps, *Misidopsis bahia*

The Oppenheimer Formula was applied to the bioremediation for oil spill from the Mega Borg in the Gulf of Mexico. A series of acute toxicity tests using Mysid shrimps, *Misidopsis bahia*, were undertaken by using the actual water samples from this remediation site.

A course of breeding tests using Mysid shrimps was undertaken by adding the formula at various concentration in three sets of experimental water samples with Mysid shrimps; a control, a non treated and a treated samples.

For the result, after 96 hours breeding tests, no LC₅₀ of Mysid shrimps was recorded in any experimental sample sets or at any concentrations of microbial products. A few demise of the shrimps were observed during the experiments, however, no correlation between these demises and concentrations of added microbial products was identified. Given that the growth condition of the Mysid shrimps in the control sample was observed to be fine, these shrimps used for this breeding test were considered to be suitable for the series of experiments.

These above results demonstrate that no toxicity of Oppenheimer's formula onto Mysid shrimps was identified.

Reference Environmental Research Institute, US EPA, July 1990.

3) Evaluation of Oppenheimer's formula in cultures of the rotifer *Brachionus plicatilis* Müller

A series of bioassays was undertaken at The University of Texas Marine Science Institute to test the effects of Oppenheimer's formula (hereinafter referred as 'the formula'), using as test organism the rotifer *Brachionus plicatilis* Müller.

Test description

Testing under conditions restricting the presence of microbial contaminants. Rotifers cysts were disinfected by a 3 minutes immersion in sodium hypochlorite at 0.5% concentration. Cysts were hatched in sterile seawater at a salinity of 15 ppt under constant light. Rotifers were transferred under aseptic conditions to 50 ml test tubes with screw caps, at a final density of 5 rotifers per ml. The formula was added at 0.01mg per ml to four culture tubes. An aliquot of unfiltered seawater was added to each of the four control cultures as a source of bacteria. After 4 days of culture, four samples were taken from each culture and the rotifers counted. The average count was multiplied by ten to obtain the density of rotifers per ml. Rotifer production per ml and per day (P) and rotifer growth rate (GR) were calculated as follows and these two data were analyzed by one-way ANOVA, using the computer program Statistix II;

$$P = (\text{final density} - \text{initial density}) / \text{days of culture} (4)$$

$$GR = (\ln \text{ final density} - \ln \text{ initial density}) / \text{days of culture} (4)$$

Testing under condition not restricting the presence of microbial contaminants. Rotifers were resuspended in filtered seawater (1.2:1) after washing by screen and transferred to 100 ml beakers. The experiment was run twice. Initial rotifer densities were 30 and 17 ml⁻¹ in the first and second run, respectively. The formula was added to four beakers as described above. After four days of culture, P and GR were determined.

Conclusion

The addition of the formula to cultures of rotifers did not cause any adverse effects on daily rotifer production nor in growth rate over a period of four days of culture under conditions either restricting or not the presence of microbial contaminants.

4) Fertilization tests on Echinus, *Toxopneusts pileolus*

A series of fertilization tests on eggs of Echinus, *Toxopneusts pileolus* was undertaken to determine effects of Oppenheimer's formula on living organisms. Such fertilization tests are used by Canadian EPA and US EPA in bioassays for aquatic lives in seawater.

Oil dispersant and household neutral detergent were also tested for the purpose of comparison.

Oppenheimer's formula, oil dispersant and household neutral detergent were respectively added in seawater at concentrations of 0 ppm (control), 1 ppm, 5 ppm, 10 ppm, 50 ppm and 100 ppm respectively. Formation of fertilization envelop on 100 eggs of Echinus in each samples were observed and those population were counted using microscope. Experiments were run four times and an average value of each obtained numbers was determined as a fertilization rate.

Fertilization rates on Echinus eggs

Concentration ppm

Control	Oppenheimer's formula	Oil dispersant	Household neutral detergent	
0	95%			
1		96%	78%	82%
5		92%	61%	20%
10		92%	41%	6%
50		80%	13%	1%
100		68%	4%	0%

As shown in the table above, the fertilization rates for both Oppenheimer's formula, oil dispersant and household neutral detergent declined as concentration of each experimental waters increased. However, degrees of decline rates varied significantly among them. Particularly, in 100 ppm, while oil dispersant and household neutral detergent disturbed the fertilization in notable degree (4% and 0%, respectively), the formula affected less (68%). Based on these results, effects of the formula are considered to be smaller than oil dispersant and household neutral detergent.

Bioremediation on the shore after an oil spill from the Nakhodka in the Sea of Japan, II. Toxicity of a bioremediation agent with microbiological cultures in aquatic organisms, Marine Pollution Bulletin 40 308-324(2000)

Impact on plants

Growth disturbance tests on Algae, *Skeletonema costatum*

A series of growth disturbance tests of Oppenheimer's formula on Algae, *Skeletonema costatum* was undertaken as described below;

The formula was added in each water samples (mixture of crude oil and seawater; proportion was 10% and 90% respectively) at various concentrations (except 'Seawater sample' and 'Oil containing sample') respectively. Algae were transferred to each experimental water samples at 10,000 - 20,000 cells per ml as initial level and cultured for 7 days. The growth curve obtained through the tests is shown in the graphics below;

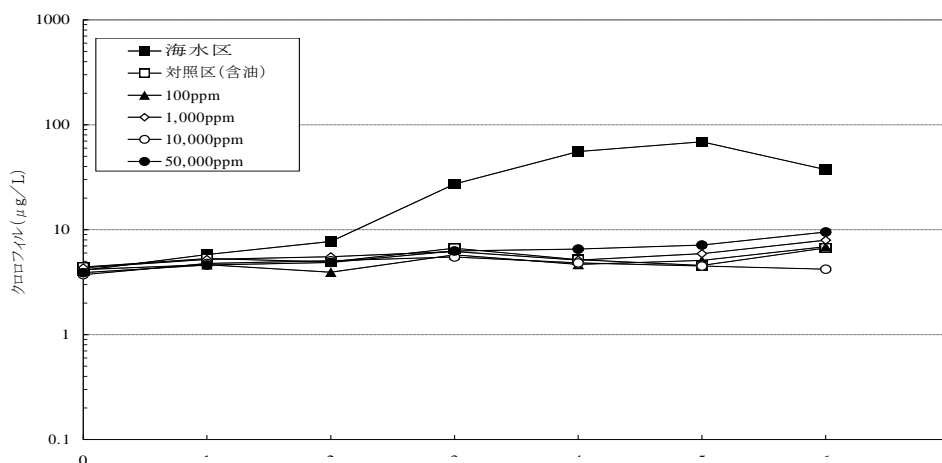


Figure : Growth curve of Algae (Chlorophyll a)

Since 'Seawater sample' shows a normal growth curve, the algae used in these experiments were confirmed to be suitable for this experiment purpose. No significant difference between growth curves for 'Oil containing sample' and other experimental samples where the formula added were identified.

Based on the results, toxicity of the formula in coexistence with oil (crude oil) was determined to be same or lower level than it of oil which exist solely. No growth disturbance of Algae was observed even at the maximum level of concentration at dissolution limit of formula, and its value of EC₅₀ showed over detection limit.

Environmental research technology section, Planning and Coordination Bureau, Environmental Agency: A study on Guideline for usage of bioremediation for oil spills in seashores (Working group for a guideline of bioremediation application for oil spills at sea, A research on Safety evaluation methodologies for remediation using micro organisms, Case studies on application of bioremediation to oil spills, 1998 - 2000).

Eco toxicological assessments by bio assays

[Microorganisms never generate toxin.]

Given that there are some potential risks that hazardous substances are generated as byproducts on process of bioremediation, several bioassays on our microbial products have been conducted to determine whether such hazardous byproducts are generated on degradation processes.

Bioassays are known as analysis methodologies that evaluate toxicity of an unknown chemical substance, as a mass, in the environment by using living organisms.

1. Mutagenicity test, 2. Endocrine disturbing action test, 3. Umu test and 4. Growth observation test of killifishes have been undertaken as bioassays of our microbial products.

An evaluation of treated groundwater contaminated by trichloroethylene in bioreactors, No.1. Mutagenicity test and Endocrine disturbing action test using field water samples after treatment by bioremediation on-site

An evaluation of treated groundwater contaminated by trichloroethylene in bioreactors, No.2. Mutagenicity test and Umu test using experimental water samples after treatment by bioremediation test

An evaluation of treated groundwater contaminated by trichloroethylene in bioreactors, No.3. Growth observation test of killifishes

An evaluation of contaminated groundwater treatment by bioreactors (trichloroethylene contaminated groundwater) , No.1.

(Mutagenicity test and Endocrine disturbing action test using field water samples after treatment by bioremediation on-site)

Summary

Complex contaminated groundwater with high concentration of VOCs such as trichloroethylene (200 - 500 mg/L) and Cis-1,2-dichloroethane (100 - 300mg/L) was treated by microbial products and a bioreactor system. Removal ratio of the VOC contaminants from this treated water was recorded as of 99%. No hazardous byproducts were identified in this treated water according to the results from the bioassays.

Analysis description

Mutagenicity test

Endocrine disturbing action test by yeast two-hybrid study

Analysis procedure

Samples One water sample taken from effluent of bioreactor system, 350 L (Sampling date 2001.11.26), and one water sample taken from effluent of bioreactor system, 1 t (Sampling date 2002.2.4)

Pre-treatment, concentration and cryopreservation (-20) of the samples were provided within 24 hours of sample arrival.

Mutagenicity test

The samples were analyzed by Ames technique Maron and Ames, 1983 using *Salmonella typhimurium* TA98 and TA100 to determine mutagenicity of substances in the samples. Sample products in the market place (Oriental Yeast Inc.) was used as S9 which is used for metabolic activation of indirect mutagen, and prepared as S9 mix by adding coenzyme.

Yeast two-hybrid study

An yeast two-hybrid study which was developed by Nishihara<Nishikawa?> (Osaka Uni.) et al was provided for the samples to screen presence of endocrine disturbing action (similar to estrogen action). *Saccharomyces cerevisiae* PGBT9-ER ∇ HPGAD424-TIF2 was selected as strain of this study. This strain is yeast made by human estrogen receptor with co-activator. Details of this study procedure was conformed to the methodology by Nishihara et al Nishikawa *et al*, New screening method for chemicals with hormone receptor with coactivator, Toxicology and Applied Pharmacology, 154 76-83. 1999 .

Conclusion

Mutagenicity test:

No mutagenicity to both TA98 and TA100 was identified in the samples, regardless of S9 existence.

Yeast two-hybrid study: Sample No.1 and No.2 were studied for screening of endocrine disturbing action (similar to estrogen action). Results showed that no such estrogen similar action (β -gal action) was identified.

References: Soil and environment laboratory, Agricultural Dept. Saga Uni., Report of Mutagenicity test and Endocrine disturbing action study for the effluent from a bioreactor system, March 2002. Soil and groundwater environment group, Soil environment section, Water environment department, Environmental management bureau, Ministry of Environment, Report; Research, development and promotion of general purpose groundwater remediation device, 2001.

An evaluation of contaminated groundwater treatment by bioreactors (trichloroethylene contaminated groundwater) , No.2.

(Mutagenicity test and Umu test using experimental water samples after treatment by bioremediation test)

Summary

Contaminated groundwater including trichloroethylene (10 mg/L) was treated by microbial products (bioreactor test and soil batch test). The samples after this treatment process were tested for Mutagenicity and Umu testing (Umu test is used for safety evaluation of discharge water in Germany). All analysis results showed negative and no toxin in byproducts on degradation process were identified.

Analysis description

Mutagenicity test, Umu test

Analysis procedure

Samples Effluent from bioreactor (after the treatment)

Soil batch sample (after the treatment)

Mutagenicity test Test methodologies stipulated in 'Hazardous evaluation standards'(Ministrial declaration No.77 and No.67 by Ministry of Labor, Japan) and 'Concrete methods and evaluation techniques of results for Mutagenicity tests using microorganisms' was partially selected for this experimental test.

Umu test Umlac (Japan Antibody Institute Inc.) test kit was used for this analysis.

Conclusion

Mutagenicity test Samples treated by bioremediation demonstrated no upward trend in its number of reversion colony and therefore it was determined that the mutagenicity in these samples was negative. Besides, no growth inhibitory effect or sedimentation of test material was observed. Based on above results, the samples after treatment under this experimental condition are considered to have no revision mutagenicity.

Umu test Given that no mutagenicity by Umu test for soil and groundwater samples after treatment was observed, the treated samples were considered to be safe for living organisms.

References : Chemicals Evaluation and Research Institute, Final report for a Mutagenicity test using microorganisms SH-02 (MSDS). A consortium research project for immediate effectively rebirth of community for fiscal 2001, Final report, 'Development of efficient bioremediation technologies for contaminated soil and groundwater using consortiums of microorganisms', March 2003.

**An evaluation of contaminated groundwater treatment bybioreactors
(trichloroethylene contaminated groundwater) , No.3.**
(Growth test of killifishes, *Oryzias latipes*)

Summary

Contaminated groundwater (Trichloroethylene, 10mg/L <mass concentration) was treated by a bioreactor system. Safety of the treated water was evaluated by observing life cycle of killifishes, *Oryzias latipes*.

Evaluation procedure

Samples	'TCE'	Contaminated water with 10mg/L of TCE
	'Treated'	Treated water
	'Tap water'	Tap water

Description Incubation, survival ratio and change of living environment associate with killifishes (*Oryzias latipes*) in above described three types of water were respectively tracked. Spawning test and anatomy for the adult fishes were conducted to obtain those liver index, genital gland index and measure level of VTG in blood. An evaluation for safety of treated water was obtained by examining of these data (see Figure in next page).

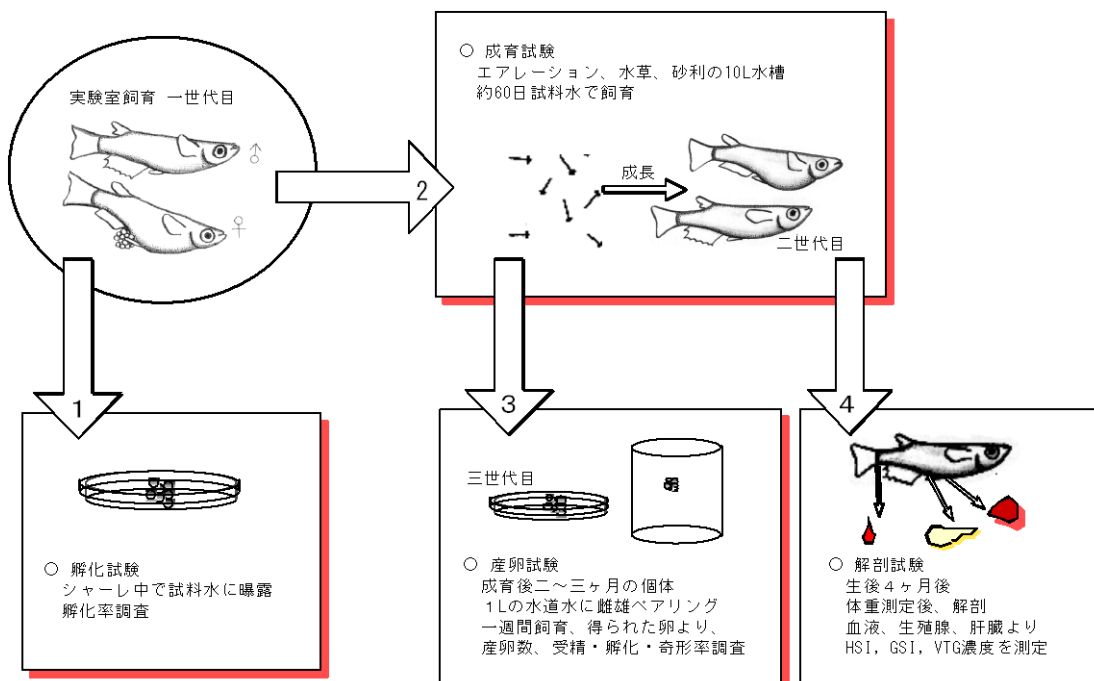


Figure: Safety evaluation for ecosystem, Flow chart of grow test using killifishes (*Oryzias latipes*)

Conclusion

In the course of the test, significant changes such as a decrease trend of survival ratio on the killifishes (*Oryzias latipes*), generation of black alga in the fish tank and an increase trend of their liver index were observed in the 'TCE'. Contrary, such changes were not observed in the 'Treated' as well as 'Tap water' which has no impact on living environment for killifish. Through this observation of killifishes' life cycle, herewith, the safety of treated water by the bioreactor system was considered to be confirmed.

Reference: Faculty of Environmental and Symbiotic Sciences, Kumamoto Prefectural University, A safety evaluation of treated groundwater by a general-purpose treatment device for complex contaminated water by an observation test using life cycle of killifishes (*Oryzias latipes*), A consortium research project for immediate effectively rebirth of community for fiscal 2001, Final report, 'Development of efficient bioremediation technologies for contaminated soil and groundwater using consortiums of microorganisms', March 2003.

5.Kinetic analysis of microbial cultures in bioremediation

[Microbes will die when they eat up their food [oil].]

Measuring total number of all microorganisms and oil-degradable ones in an oil-contaminated soil sample showed that oil eating microorganisms decreased along with the reduction of oil.

Added microbes don't have a negative impact (such as destruction) to native microbes, instead, they coexisted with other microbes in the surrounding area.

Two sets of each system were made and concentration of oil, microorganism population and structure of microorganism culture in all systems were respectively observed.

Analysis description

Oil concentration: Carbon tetrachloride extraction, FT-IR method

Total bacteria population: Direct microscopic count method (EB staining method)

Oil degrading (petroleum assimilating) microorganism population: Petroleum DVC method

Microorganism culture observational study: PCR-DGGE method

Results

(1) Both populations of total bacteria and oil degrading bacteria in 'Augmentation' demonstrated highest level among all experimental systems after 14 days. However, these populations declined gradually to the same level of 'Stimulation' and 'Catalyst addition' after two months. Added microorganisms were also observed to be declining after completion of oil degradation.

(2) No differences in banding patterns of microorganism cultures were observed in any of experimental systems where nutrients were added. This result indicates that additive microorganisms give no impact on indigenous ones (ones that increase by stimulation).

(3) Highest ratio of oil degradation effect was observed in 'Augmentation'.

Conclusion

This study demonstrated that no differences between stimulation and augmentation treatments that impact on ecosystem of indigenous microorganism. Also, no effect on its ecosystem by additive microorganisms in the augmentation was identified. A possibility was indicated in this study that these additive microorganism cultures may build consortia of microorganisms including indigenous ones that promote together degradation of oil.

This collaborative study was made with Institute of Agricultural and Forest Engineering, University of Tsukuba sponsored by Showa Shell Sekiyu Environmental Research Support for fiscal 2001.