Risk management of GEMs intensively-released for bioremediation

2004.7.1

Masanori FUJITA

Department of Environmental Engineering
Osaka University

Environmental Biotechnology
--a general view--

(1) Biotreatment
municipal/industrial waste and wastewater

(2) Bioremediation
soil, sediment, groundwater, coastal zone
cf. phytoremediation/ ryzosphere

(3) Biomonitoring/Bioassay
genomics
proteomics
metabonomics

(4) Biorecycle/Biorecovery
- compost
- biogas
- biodiesel
- ethanol
- lactate/biopolymer

(5) Biocontrol
- biocorrosion
- biofouling
- sterilization
- pasteurization

(6) Biofertilizer/Biopesticide

Possible field of intentional release of effective microorganisms (natural occurring) and /or genetically engineered microorganisms (GEMs)

(1) Biological treatment of wastewater

(2) Bioremediation

(3) Compost/Biogas

(4) Biofertilizer/Biopesticide

General view of GM food
Socio-economics consideration

Benefit to whom?

(1) Consumer: No benefit?

Lack of economical and nutritional benefit

Lack of scientific evidence/uncertainty

(Appearance of new hybrids? safety of GM food?)

Food culture

Unacceptable of GM Foods

(2) Farmer(Producer):Great risk?

Opaque of benefit?(Due to the way of Japanese agriculture?)

Appearance of new hybrids? Adverse effect to biodiversity?

Following the consumer's choice

Unacceptable of GM plants-cultivation in Japan

Biosafety or Biorisk on intentional release of microorganisms (Both natural occurring and GEMs)

→affected by discussion on GM food

Conservation of biological diversity and forest resource

1992 : Earth Summit at Rio de Janeiro
(Rio Declaration on Environment and Development, Agenda 21, Biodiversity treaty, etc)

2000 : Cartagena Protocol on Biosafety

2002 : Earth Summit at Johannesburg
(World Summit for Sustainable Development)

2004.2.19
: Law for conservation of biodiversity by regulating use of Living Modified Organisms

Examples of Living Modified Organisms (LMOs)

red bean, soybean, strawberry, sugar beet, melon, rice, corn, lettuce, tomato, cauliflower, cucumber, wheat, papaya, potato, broccoli, rape

alfalfa, petunia, tobacco, bent grass, carnation, cotton, torenia, chrysanthemum, zoysia

Bt-corn, Bt-cotton, Bt-potato (Bt : *Bacillus thuringiensis*)

golden rice : carotene producing rice (Vitamin enriched rice)

50 % of soybean is LMO, and 95 % of consumption in Japan is covered by imported soybean.

In this lecture,
I focus on risk on environmental release of GEMs

Utilization of GEMs and/or effective microorganisms
to solve the environmental problem

Risk of environmental release of GEMs and/or effective
microorganisms (natural occurring)

Question?
Why do we manage the risk due to utilization of GEMs?

Requirement for rapid, safe and cost-effective *in situ* Bioprocess

Application of GEMs and/or natural occurring (Bioaugmentation) to

Biotreatment, Bioremediation, Compost, Biofertilizer, Biopesticide, and so on.

Development and utilization of GEMs to enhance *in situ* Bioprocess

Focus on Bioremediation in this lecture

Structure of GEM and biological risk assessment for the environmental release of GEMs

Recombinant(GEM) -- risk
Host -- risk
Vector -- risk
Exogenous gene -- risk

Cf. Self cloning

Concept of risk management after the environmental release of GEMs (bioaugmentation)

Monitoring of released GEMs and horizontal gene transfer (by conjugation)

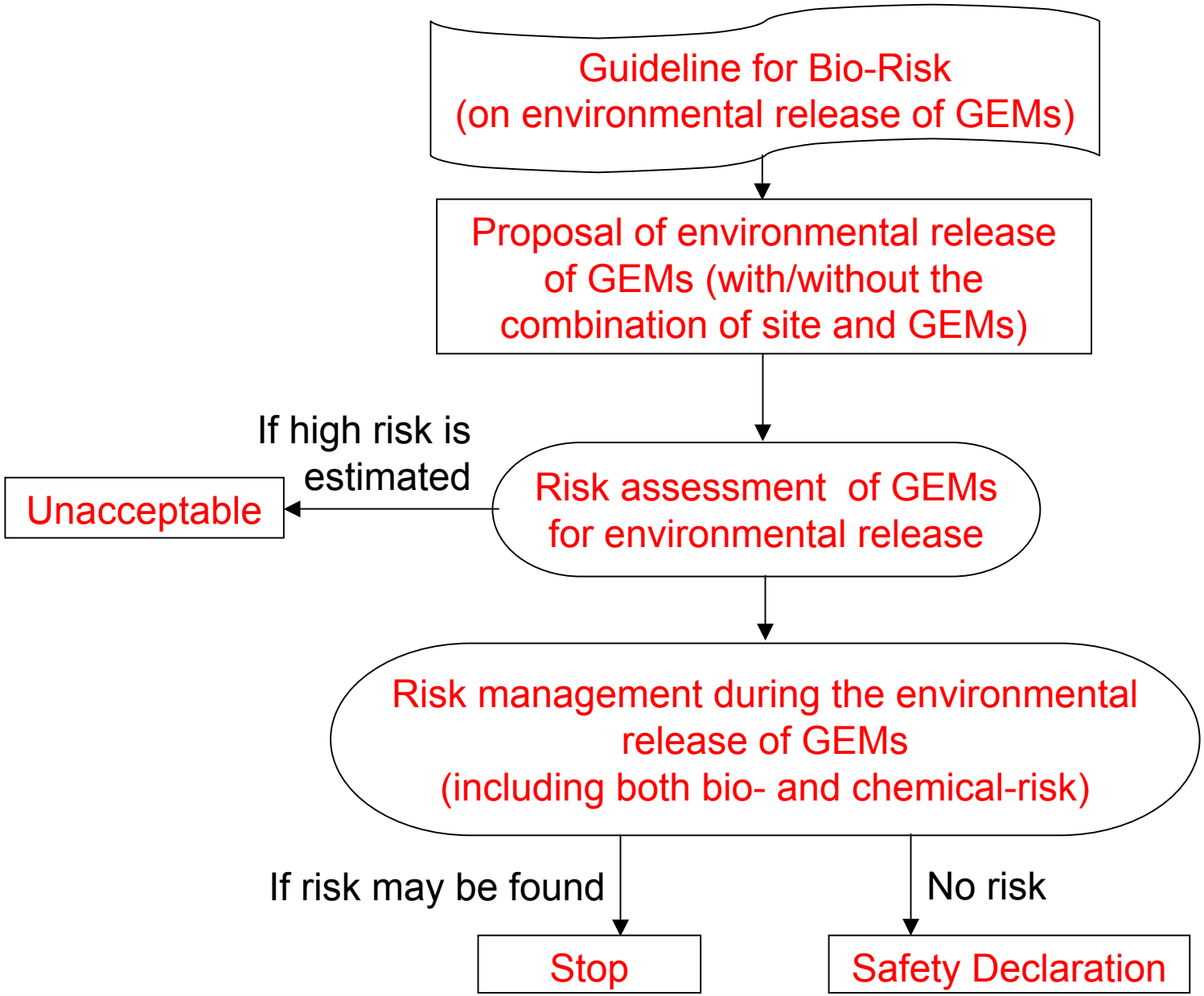
(Survival of GEMs, Appearance and growth of transconjugants)

Scientific evidence on expected risk

Risk assessment based on scientific evidence

Risk management during *in situ* Bioremediation
(stopping immediately, end of bioremediation, end of risk management, etc)

Procedure for risk assessment and management for environmental release of GEMs



Expected risk

Harmful effect to higher organisms including human being as a result of horizontal gene transfer to/from indigenous pathogens

Adverse effect to Biological diversity and/or ecological functions such as biogeochemical cycles

Harmful effect to higher organisms by environmental release of GEMs (=Pathogenicity)

Risk assessment before application

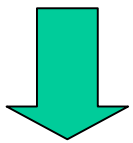
(A) Pathogenicity of the host (guideline reference)
(B) Genetic information on vector
(C) Genetic information on genetically engineered DNA sequences (GEDS) such as recalcitrant compound degrading genes, heavy metal resistant genes, and antibiotics resistant genes
(D) Produced GEMs

Risk management

(A) Survival of GEMs
(B) Secondary GEMs by acquiring pathogenic genes from indigenous pathogens
(C) Transfer of GEDS including antibiotic resistant genes to indigenous pathogens
(D) Risk management based on scientific risk assessment

Harmful effect to biological diversity and ecological functions

Targeting microbial community responsible for specific functions such as material circulation (C, N, S, etc) and energy flow



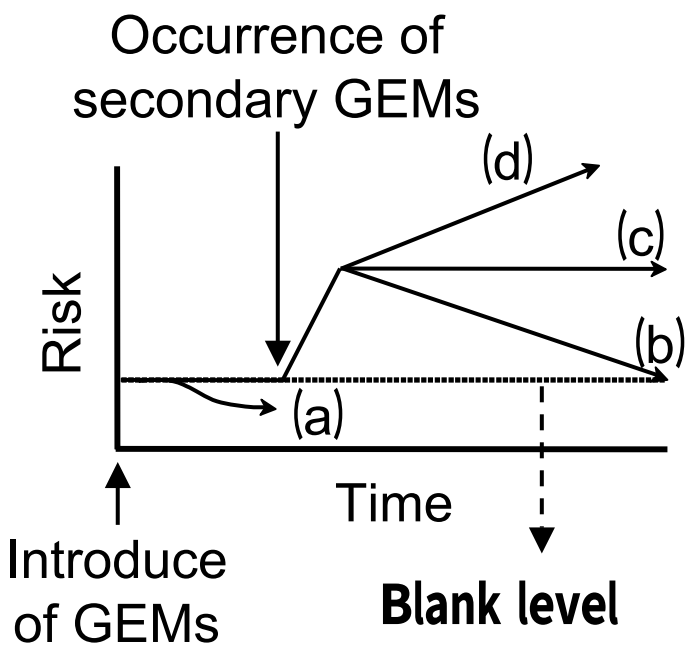
Occurrence of secondary GEMs by acquiring GEDS with/without gene rearrangement through conjugative transfer

Possibility of the adverse effect to
biological diversity and ecological functions

Main factors lead to risk occurrence, and the pattern of the time course of risks

Factors

- Characteristics and survival of the GEMs
- DNA transfer with/without rearrangement from indigenous microbes to GEMs
- Transfer of GEDS with/without rearrangement from GEMs to indigenous microbes
- Survival of secondary GEMs
- Response of the indigenous microbial community



(a) No risk

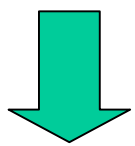
(b) Risk level decreased after occurrence

(c) Risk generally continue to be flat

(d) Risk level increased after occurrence

Objectives of this project study

- Comprehension and quantification of risk inherent to indigenous microbial community (Background risk)
  → Development of the methods and accumulation of the quantitative data
- Modeling and parameter estimation for risk assessment of environmental release of GEMs
- Accumulation of lab- and field test data for model/ parameter evaluation and risk evaluation criteria



Development of quantitative risk assessment method
and establishment of risk evaluation criteria
for environmental release of GEMs

Development of monitoring system of pathogens and functional microbes

- Investigation on DNA extraction from soil
- Investigation on specific detection of target microbes by Real time PCR
- Development of DNA microarray for detecting pathogens and other bacteria

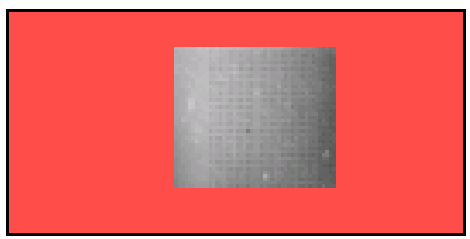
from the results of Prof. Dr. Takayuki Ezaki, Dept. of Microbiology, Gifu Univ.

Development of detection method of soil environmental pathogens

Zirconium beads beating for DNA extraction

Pouring DNA to Microplate/ Capillary containing specific PCR primers

Estimation of the number of target microbes by Real time PCR



16S rDNA fixed DNA chip for the detection and enumeration of target microbes in the samples

Phylogenetic and quantitative analysis of microbes by laser scanning of positive spots

Development of the microarray for monitoring soil environmental pathogens

Microarray was developed by fixing 16S rDNA of bacterial pathogens for human being, animals, plants, and fish and shellfish

Pathogens for human being (all the level 2 and 3); 352 species

Opportunistic pathogens; 660 species

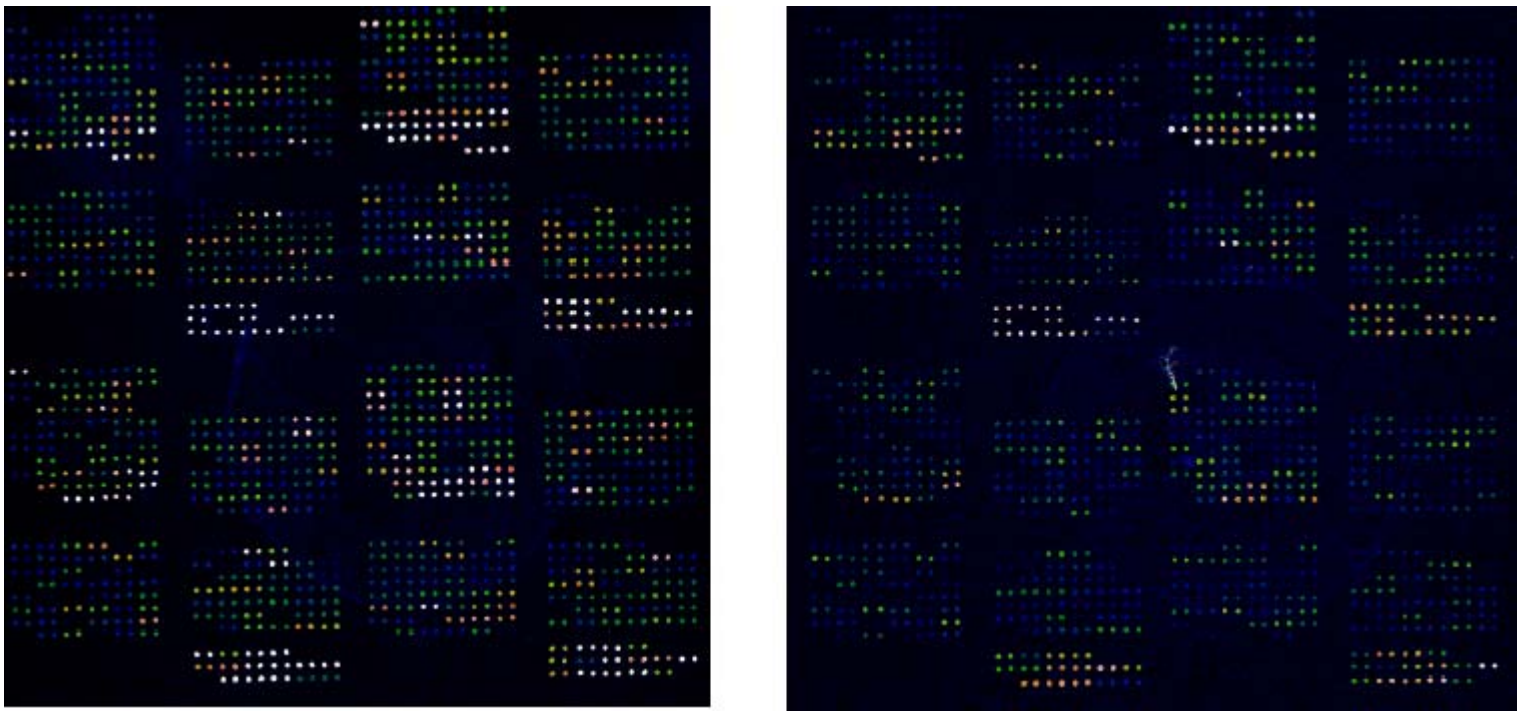
Microarray for phylogenetic analysis of soil bacteria

There exist several million species of unknown bacteria, although currently classified into 30 lineage, 1000 genera, and 6000 species.

Microarray was developed by fixing 16S rDNA of type strains listed below among known 1000 genera

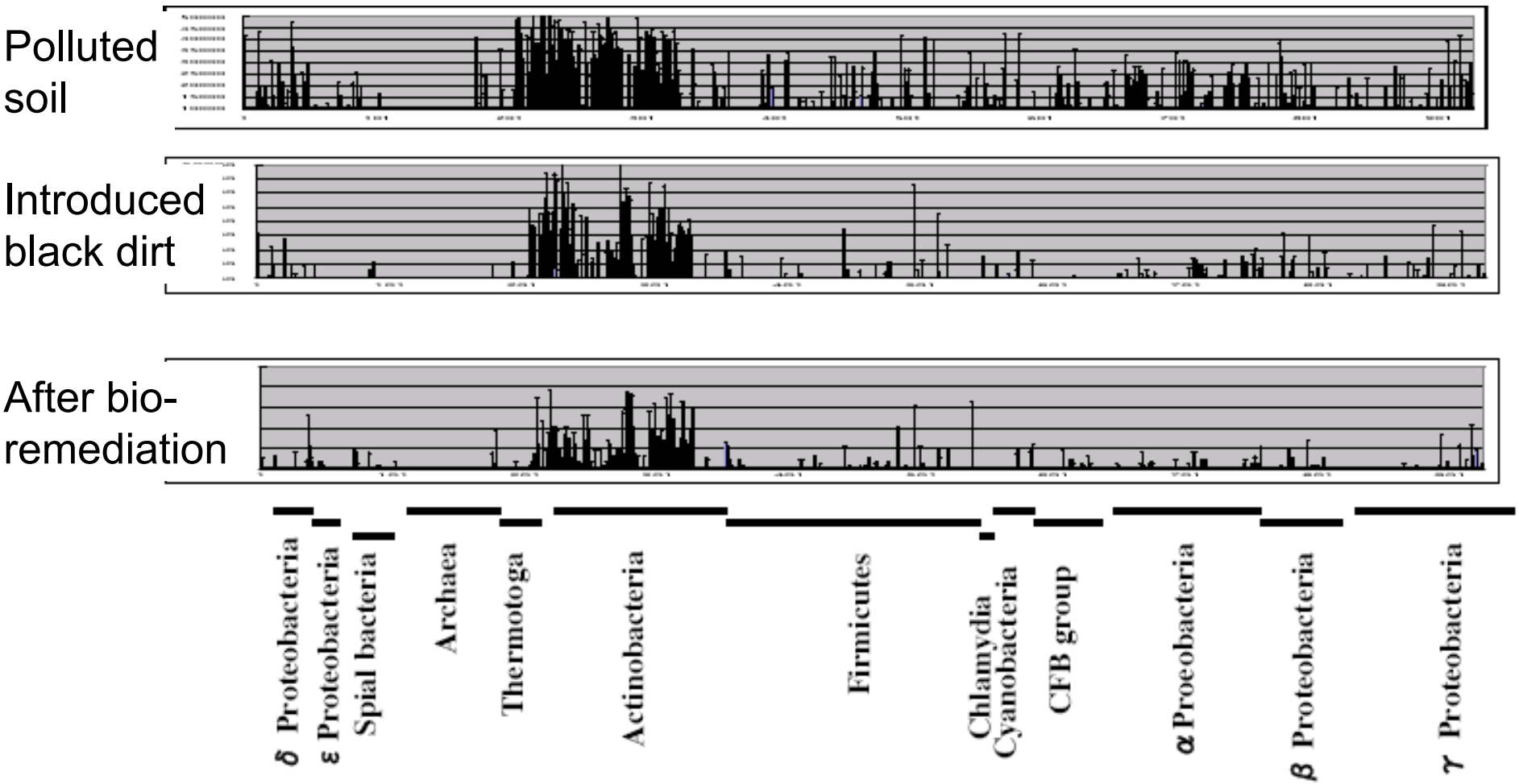
1. Archaea： 50 genera
2. Actinobacteria： 110 genera
3. Firmucutes： 150 genera
4. Cyanobacteria： 56 genera
5. CFB Group： 150 genera
6. Proteobacteria (α、β、γ、δ、ε)： 380 genera
7. Spiral Bacteria：12 genera
8. Fusobacteria： 6 genera
9. Chlamydia： 4 genera
10. Others： 15 genera

Field experiment –Phylogenetic monitoring of bacteria during bioremediation of oil polluted soil–



Polluted soil during bioremediation (Left) , black dirt used for remediation (Right)

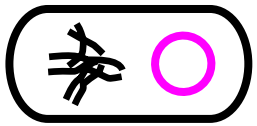
Field experiment –Phylogenetic monitoring of bacteria during bioremediation of oil polluted soil–



Risk assessment of horizontal gene transfer in the environment

- Estimation of existence ratio of mobilizer in the environment
- Estimation of the potential of environmental bacteria as recipient
- Estimation of gene transfer frequency in the environment
- Plasmid rearrangement with horizontal gene transfer

Fate of the recombinant DNA in the environment



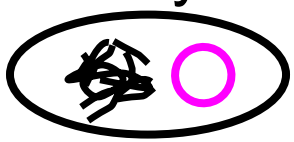
Survival of GEMs

GEMs

Horizontal gene transfer: mainly by conjugation

→ Indigenous microorganisms acquire genetically engineered DNA sequences (GEDS) with/without rearrangement by dissemination (Secondary GEMs)

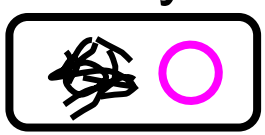
Secondary GEMs



Proliferation/survival of secondary GEMs

Horizontal gene transfer

Secondary GEMs



Proliferation/survival of secondary GEMs

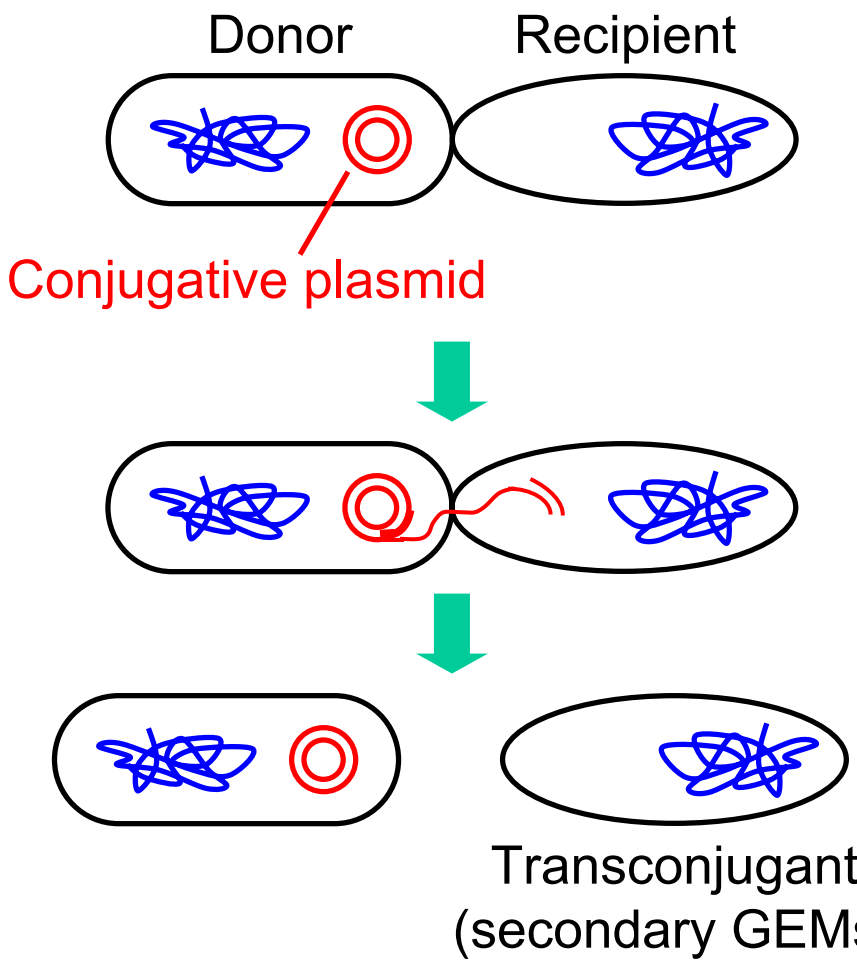
Horizontal gene transfer

Conjugative transfer in the environment

Conjugative transfer (conjugation and conjugative mediated mobilization)

➔ Most important horizontal gene transfer mechanism for plasmids

Primary mechanism which determines the fate of the recombinant DNA harbored by GEMs



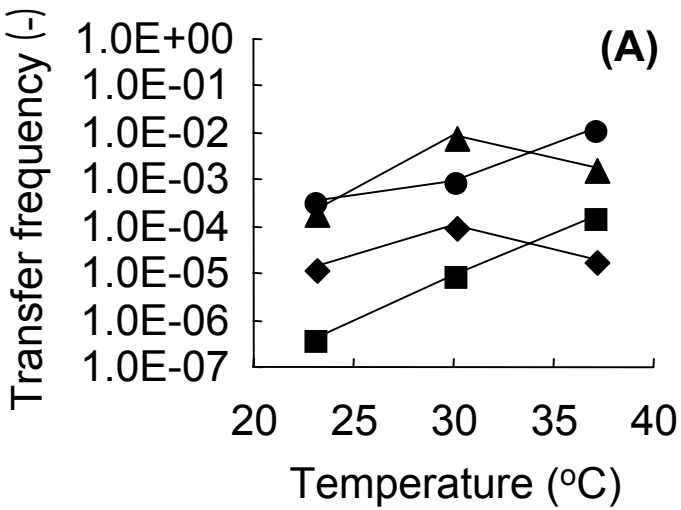
- Mechanism of conjugation -

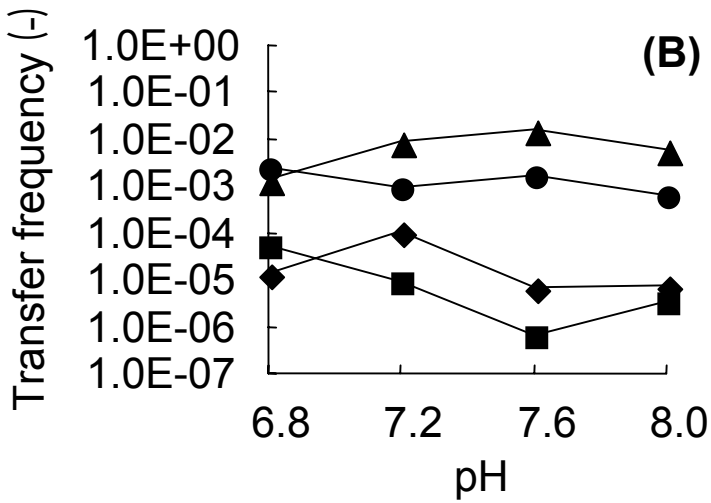
Potential of soil bacteria as recipients

Recipients (R)	Transconjugants (T) (CFU/g-wet soil) [a]	T/R (-) [a]
E. coli HB101	$3.5 \pm 1.0 \times 10^{4}$	$2.9 \pm 1.1 \times 10^{-3}$
P. fluorescens ATCC 15553	$2.6 \pm 1.3 \times 10^{4}$	$8.5 \pm 8.2 \times 10^{-6}$
***A. globiformis* NBRC 12137**	N.D. [b]	-
***B. megaterium* ATCC 12872**	N.D. [b]	-
A. hydrophila GIFU 3173	$1.0 \pm 0.3 \times 10^{3}$	$4.6 \pm 3.4 \times 10^{-5}$
***A. tumefaciens* LBA 4404**	$2.4 \pm 1.3 \times 10^{5}$	$\mathbf{2.5 \pm 1.6 \times 10^{-2}}$
S. meliloti NBRC 14782	$1.1 \pm 0.5 \times 10^{3}$	$1.4 \pm 0.6 \times 10^{-4}$

Conjugative transfer depends on host-range of the plasmid, and receptive competence of recipient itself

Environmental factors affects gene transfer frequency –Temperature/pH–





Symbol; ●: *P. putida* BH, ■: *A. calcoaceticus* AH, ▲: *Acinetobacter* sp. YAA, ◆: *Alcaligenes* sp. YAJ.

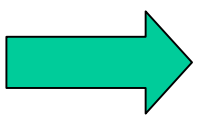
Donor : *E. coli* C600 (RP4)

Temperature affects transfer frequency, while pH around neutral is less effect

Estimation of gene transfer frequency in soil environment [a]

Recipient (R)	24h		72h	
	Transconjugant (T) (CFU/g-wet soil)[b]	T/R (-)	Transconjugant (T) (CFU/g-wet soil)[b]	T/R (-)
E. coli HB101	N.D.	-	N.D.	-
P. fluorescens ATCC15553	5.6×10^{1}	1.7×10^{-5}	2.2×10^{-1}	2.4×10^{-7}
A. hydrophila GIFU3137	N.D.	-	2.9×10^{2}	5.4×10^{-1}
A. tumefaciens LBA4404	N.D.	-	1.0×10^{2}	1.2×10^{-4}
S. meliloti NBRC14782	N.D.	-	N.D.	-

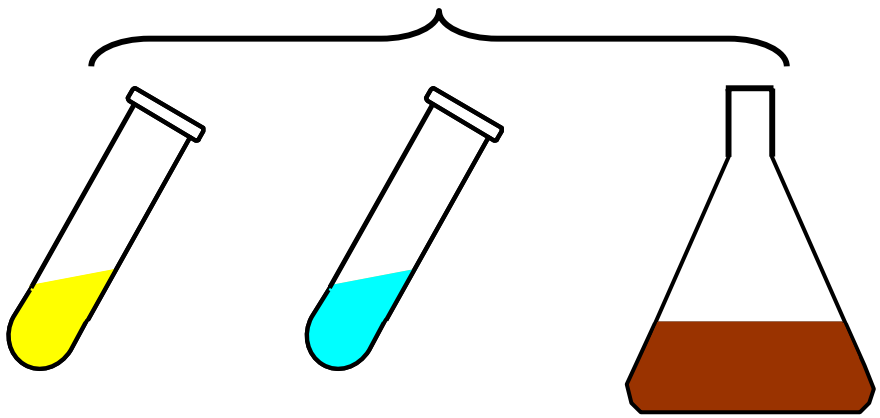
[a] Average of triplicate experiment
[b] Not detected



Less possibility of conjugative transfer in soil environment

Estimation of recipient potential : Methods

donor and recipient
(1.0 x 10^7 CFU/ml or g-wet soil)



Sterilized
- LB medium
- Environmental water
- Soil

co-incubation

for 24 h (all samples) and 72 h (soil sample) at 28 °C without shaking

Transfer frequency
= transconjugants / recipients

Bacterial strains used

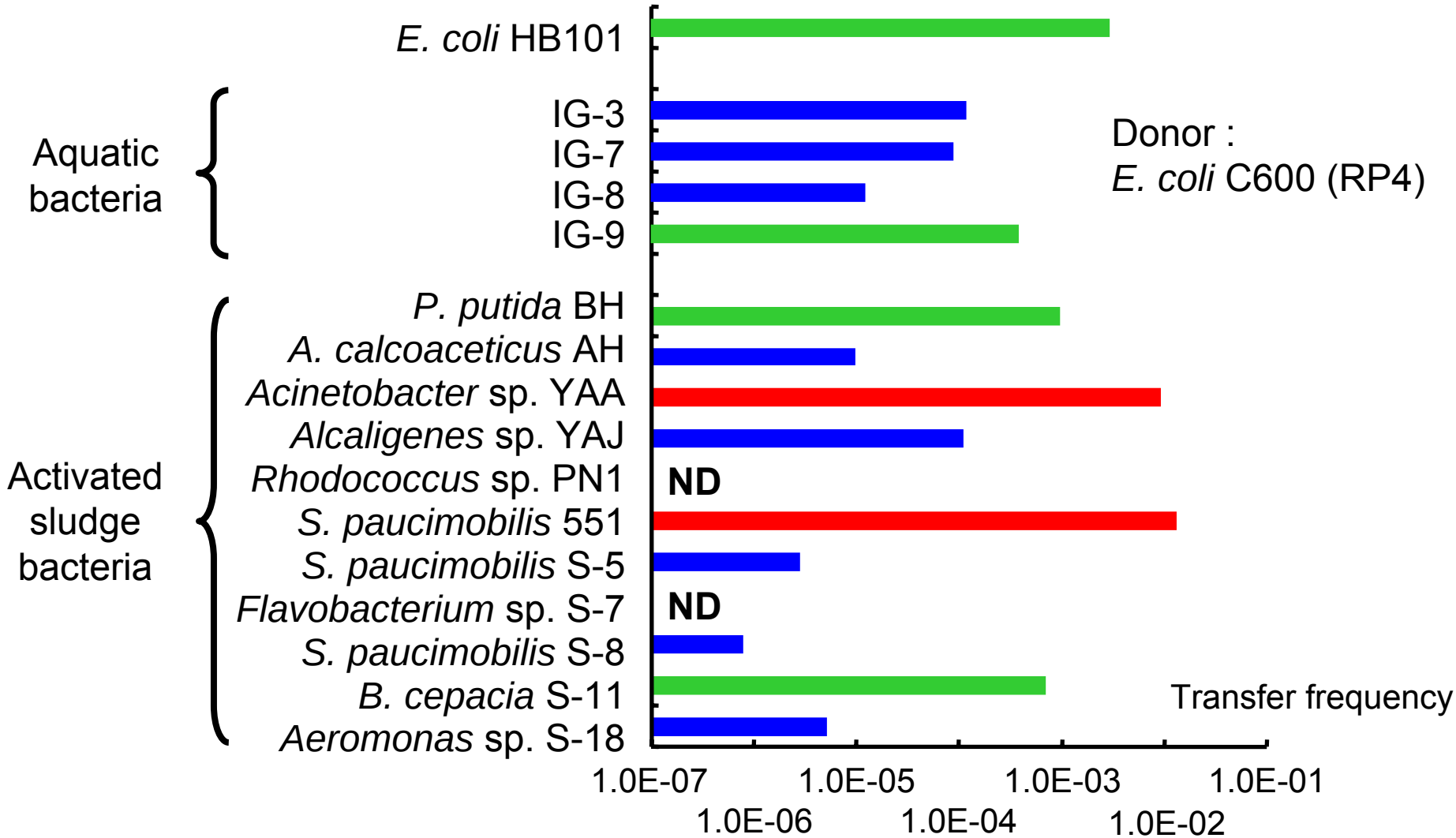
Donor

Escherichia coli C600(RP4)

Recipient

- reference
  E. coli HB101
- aquatic bacteria
  4 isolates (from river water)
- activated sludge bacteria
  11 isolates
- soil bacteria
  6 strains belonging to typical dominant species

Aquatic and activated sludge bacteria as possible recipients



► Most of aquatic and activated sludge bacteria are possible recipients

Conjugative transfer in aquatic samples

Recipient: *P. putida* BH

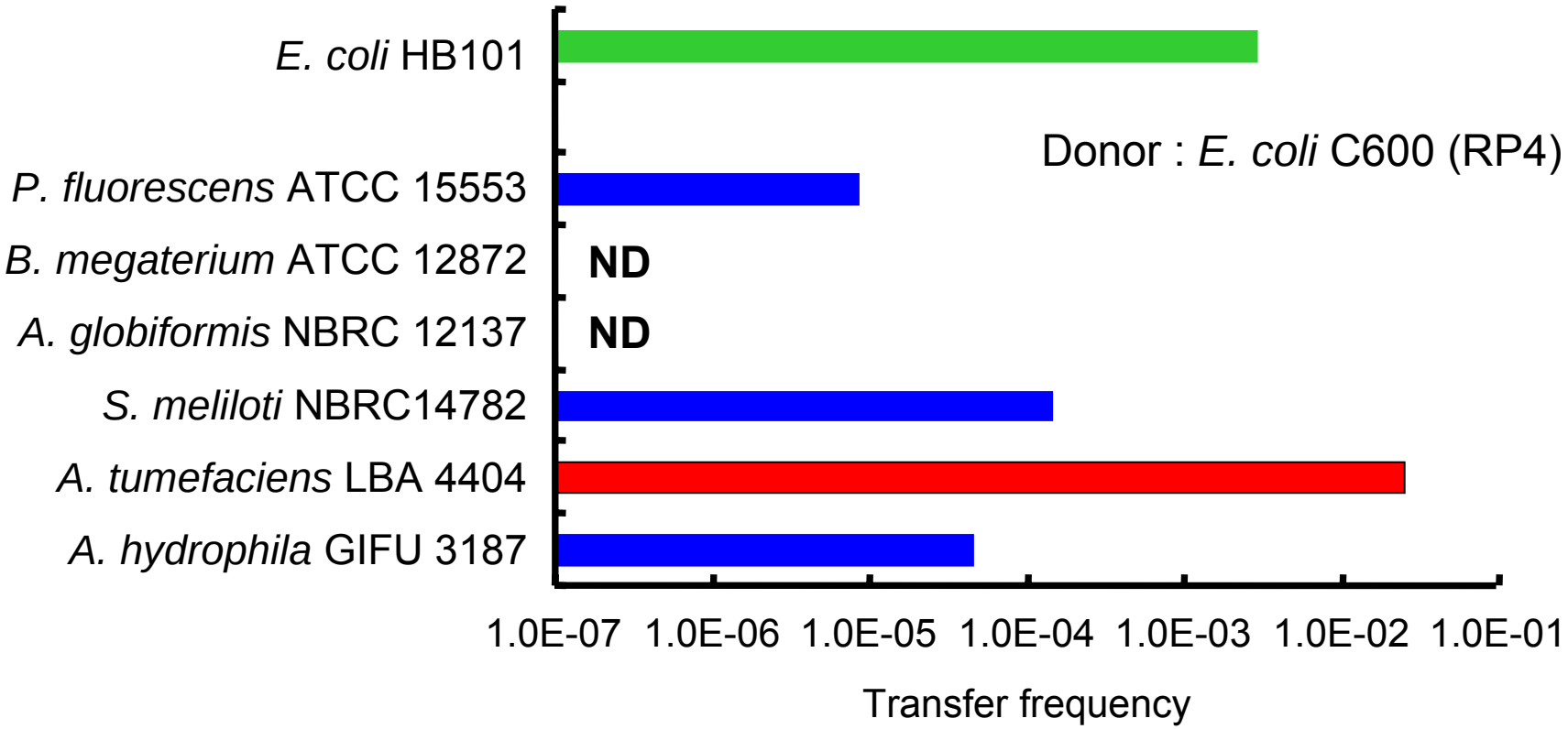
Co-incubation medium	TOC (mg/l)	Transconjugant (CFU/ml)	Transfer frequency (-)
LB medium	6,636	$4.6 \times 10^{4} \pm 5.6 \times 10^{3}$	$9.3 \times 10^{-4} \pm 8.1 \times 10^{-4}$
Influent	68.3	$6.7 \times 10^{2} \pm 4.5 \times 10^{1}$	$9.0 \times 10^{-5} \pm 4.2 \times 10^{-5}$
Effluent	21.6	$1.9 \times 10^{3} \pm 7.8 \times 10^{1}$	$5.5 \times 10^{-5} \pm 1.1 \times 10^{-5}$
Pond water1	15.8	$2.1 \times 10^{2} \pm 7.6 \times 10^{1}$	$5.5 \times 10^{-4} \pm 1.2 \times 10^{-4}$
Pond water2	51.5	$1.2 \times 10^{1} \pm 1.3 \times 10^{1}$	$6.7 \times 10^{-6} \pm 9.6 \times 10^{-6}$
River water	36.5	$3.8 \times 10^{2} \pm 4.6 \times 10^{2}$	$8.9 \times 10^{-3} \pm 1.1 \times 10^{-2}$

All the samples were sterilized before use.
Values are shown as mean ± standard deviation (n=2-3).

- Lower conjugative transfer frequency in aquatic samples than in LB medium by 1-3 orders of magnitude

  ➔ Probably due to small amount of available nutrients

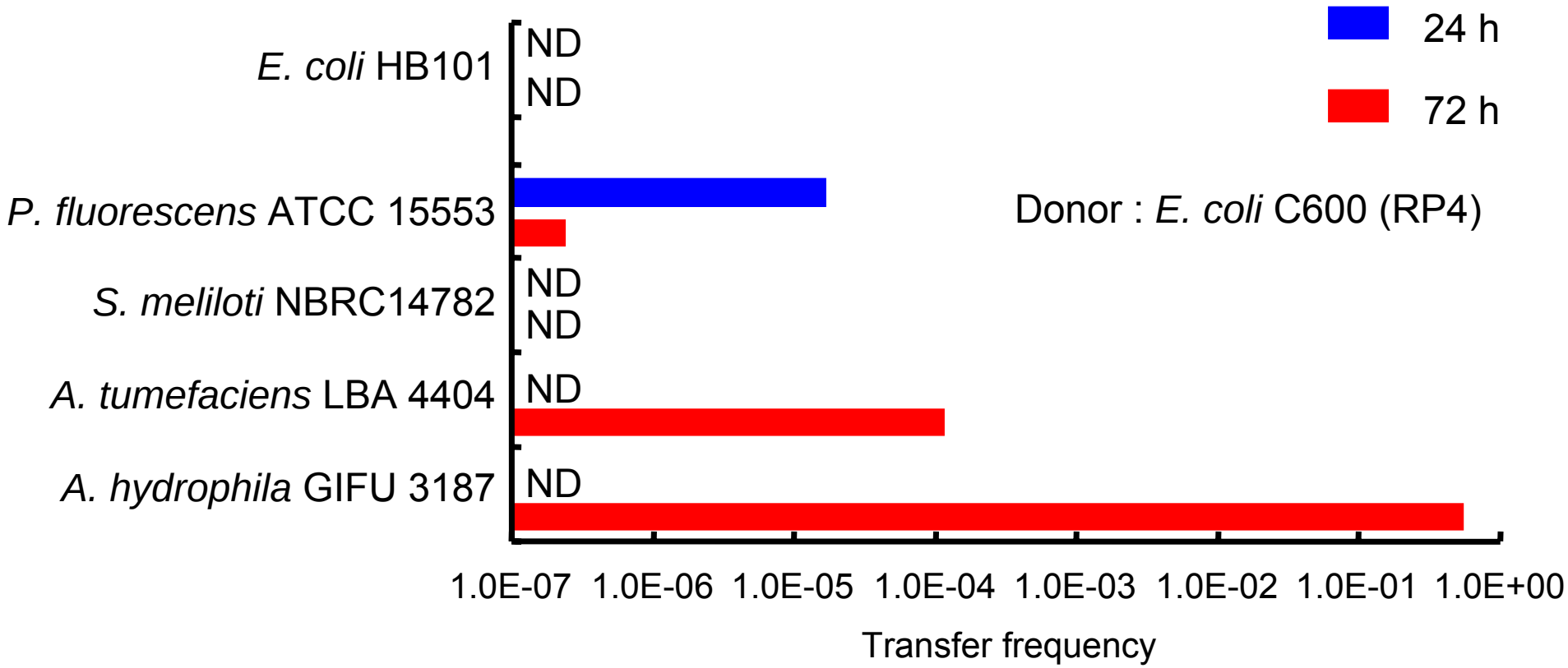
Soil bacteria as possible recipients



- Gram negative soil bacteria are possible recipients
- No gram positive bacteria could acquire plasmid RP4

➔ Depending on the host range of plasmid

Conjugative transfer in sterilized soil



- Generally smaller transfer frequencies at 24 h in soil than those in LB medium
  - ➔ Probably due to (i) low chance of cell-to-cell contact
    (ii) small amount of available nutrients

Estimation of mobilizing potential : Methods

● Detection of mobilizer and genetic analysis of mobilizing plasmids

(i) Colony PCR and phylogenetic analysis

- Target gene : *trbB*-like genes in *tra* operon
- Primers : Designed by Disqué-Kochem *et al.* (2001)

(ii) Determination of Incompatibility (Inc) group

- Target gene : *trfA* gene (IncP); *oriV* gene (IncW), *kikA* gene (IncN)
  rep gene (IncA/C)
- Primers : Designed by Gotz *et al.* (1996) and Llane *et al.* (1996)

● Measurement of mobilizing potential of isolated mobilizers

Tri-parental mating

- Donor : *E. coli* JM109(pKT230)
- Recipient : *P. putida* BH
- Mobilizer : *E. coli* C600(RP4)
  Newly isolated mobilizers (5 strains)
- Co-incubation condition:
  Filter mating on a nitrocellulose filter (0.22 μm) at 28 ^{o}C for 24 h
- Transfer frequency: Transconjugants per recipient

Relative abundance of possible mobilizers

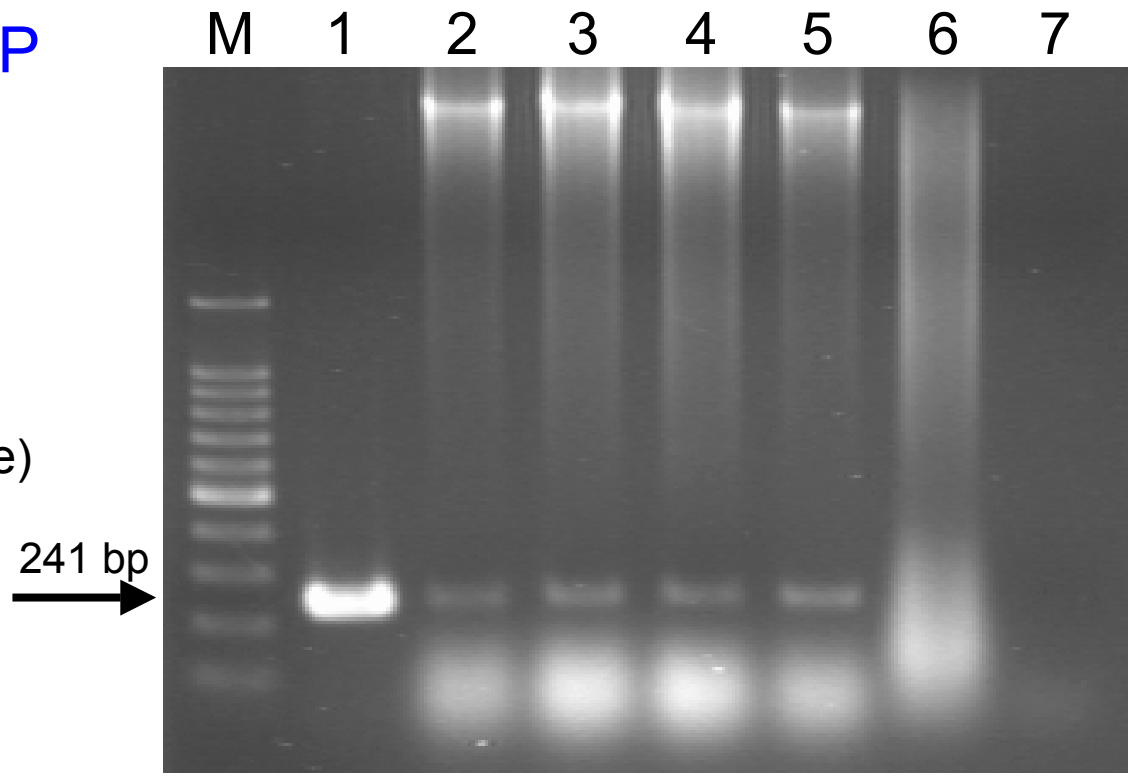
Environment	Colony PCR (%)	Taxonomical classification
Aquatic	5/263 (1.9)	*Chryseomonas luteola* (1) *Sphingomonas paucimobilis* (2) *Brevundimonas vesicularis* (2)
Activated sludge	3/68 (4.4)	*Moraxella* sp. (1) *Ochrobactrum anthropi* (1) Unknown (1)
Soil	2/240 (0.83)	*Fravobacterium* sp. (1) *Acinetobacter* sp. (1)
Total	10／571 (1.75)	-

► Mobilizers are ubiquitous in the environment especially in activated sludge

Confirmation of Inc group of plasmids in mobilizers

PCR amplification by IncP specific primers

M : 100 bp DNA ladder
lane 1 : RP4
lane 2 : MR-18
(activated sludge isolate)
lane 3 : IM-2 (aquatic isolate)
lane 4 : IM-48 (aquatic isolate)
lane 5 : IU-7 (aquatic isolate)
lane 6 : KO1-0-13 (soil isolate)
lane 7 : negative control



- Most of plasmids harbored by isolated mobilizers belong to IncP (MR-18 also showed a positive signal when used IncA/C primers)
  ➔ Possibility as broad-host-range mobilizer

Mobilizing potential of isolated mobilizer

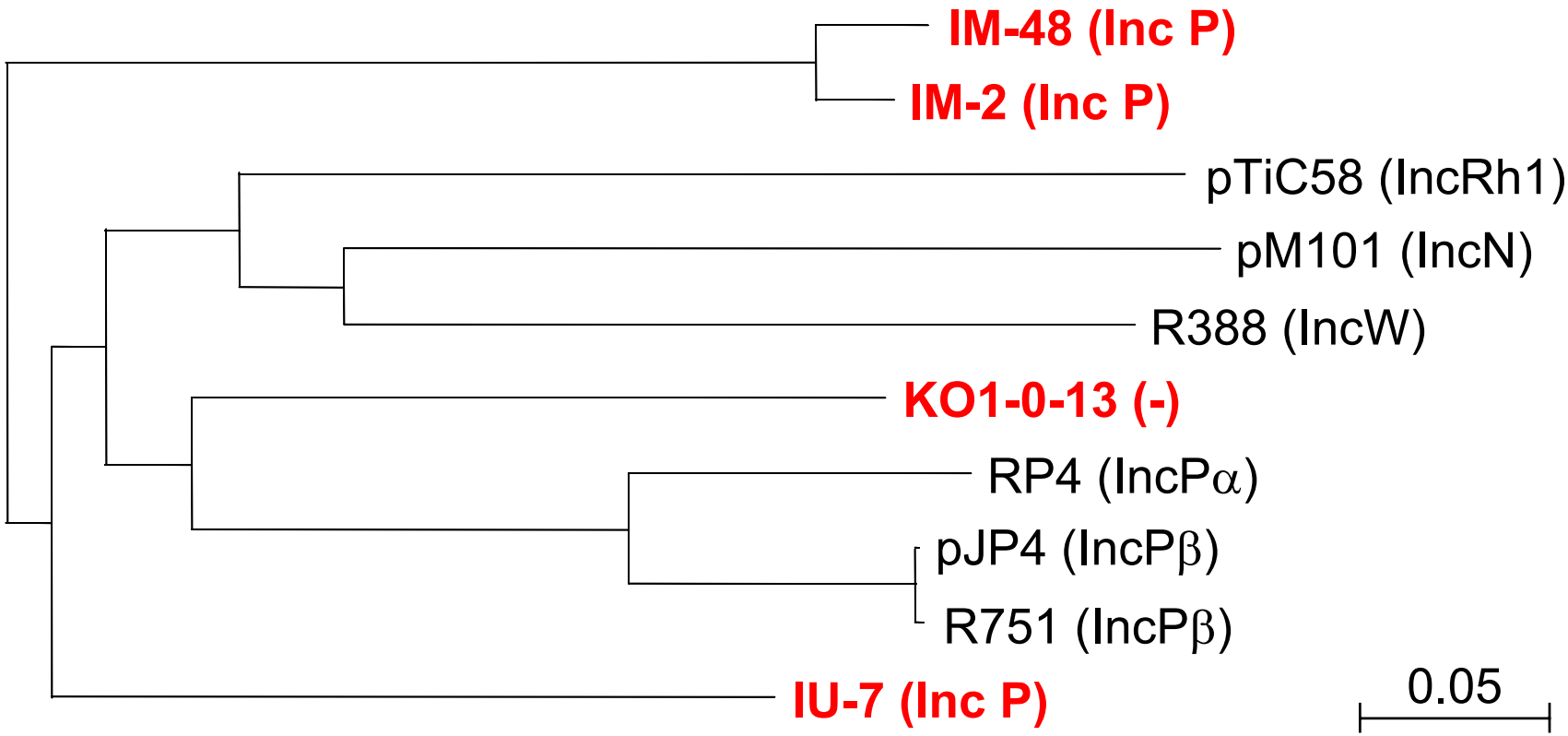
Mobilizer	Origin	Transconjugant (CFU/ml)	Transfer frequency
C600(RP4)	-	9.8×10^{5}	2.5×10^{-3}
MR-18	Activated sludge	ND	$< 3.8 \times 10^{-8}$
IM-2	Aquatic	ND	$< 1.9 \times 10^{-8}$
IM-48	Aquatic	ND	$< 2.0 \times 10^{-8}$
IU-7	Aquatic	ND	$< 5.6 \times 10^{-8}$
KO1-0-13	Soil	ND	$< 2.5 \times 10^{-9}$

ND : not detected

► No detectable mobilization by isolated mobilizers

➔ Possibility of very low frequency of conjugative transfer

Diversity of *trbB*-like genes

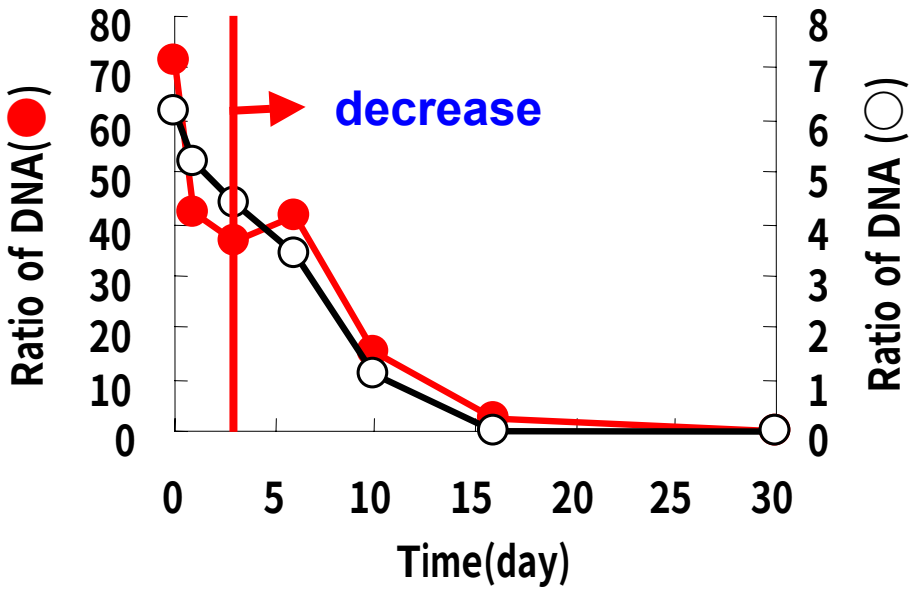


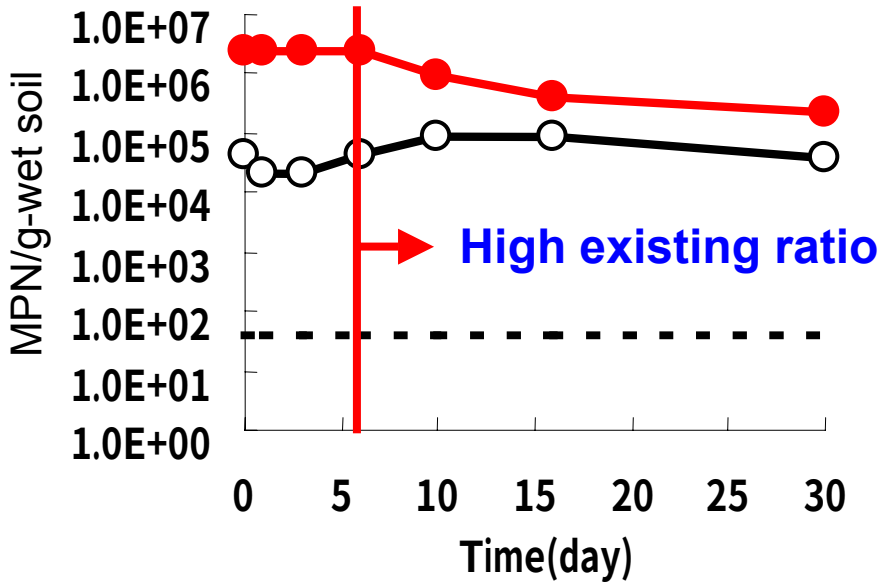
- Plasmids of most isolated mobilizers are Inc P group (possibility of broad-host-range plasmid)
- *trbB*-like genes among known broad-host-range plasmids have sequence diversity

Comprehension and modeling of the behavior of GEMs and GEDS

- Survival and persistence of GEMs and GEDS
- Occurrence frequency and survival of secondary GEMs
- Modeling of the behavior of GEMs and GEDS

Behavior of GEMs (*E. coli* C600 (RP4)) and GEDS (RP4) in soil microcosm





●Microcosm I (10^8 introduced system)　○Microcosm II (10^6 introduced system)

Introduced GEMs rapidly decreased

Possibility of the growth of transconjugant

Conjugative transfer model of plasmid

Model of the growth of *P. putida* BH (RP4) (Transconjugant:T) by transferring RP4 from *E. coli* C600 (RP4) (Donor:D) to *P. putida* BH (Recipient:R)

Model : based on mass action model of Levin *et al.* (1979)

$dD/dt = \mu_D D - kD$

$dR/dt = \mu_R R - \gamma_D DR - \gamma_T TR$

$dT/dt = \mu_T T + \gamma_D DR + \gamma_T TR$

$\mu_D = \mu_{D\max}(1 - D/D_{\max})$

$\mu_R = \mu_{R\max}\{1 - (R + T)/R_{\max})$

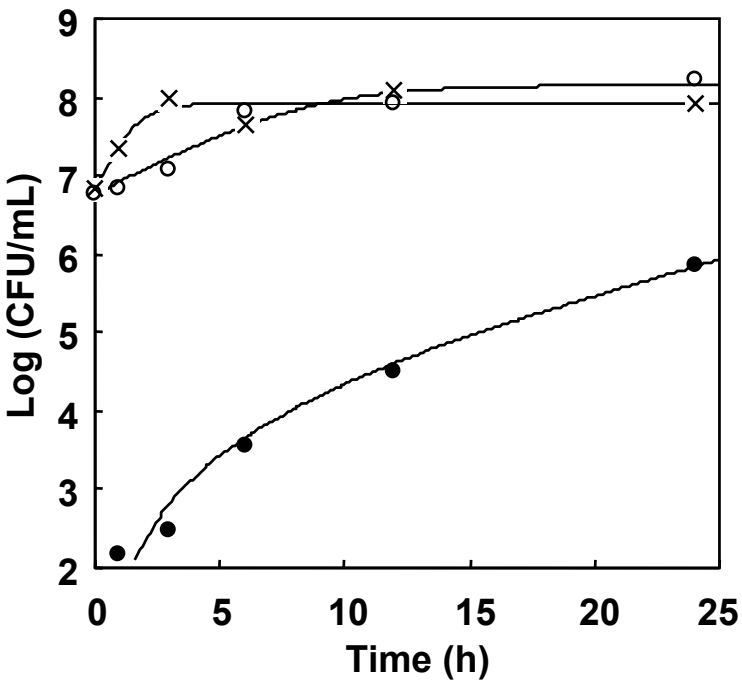
$\mu_T = \alpha\mu_{R\max}\{1 - (R + T)/R_{\max})$

t : time

D : Conc. of donor (CFU ml^{-1})

R : Conc. of recipient (CFU ml^{-1})

k : decay coefficient



Parameters	
$\mu_{D\max}$	1.6 h^{-1}
$\mu_{R\max}$	0.38 h^{-1}
$D_{\max}$	8.2 x 10^7 CFU ml^{-1}
$R_{\max}$	1.1 x 10^8 CFU ml^{-1}
α	0.48
γ_D	3.8 x 10^{-13} ml CFU^{-1} h^{-1}
γ_T	1.5 x 10^{-9} ml CFU^{-1} h^{-1}

Start ~ 10h
→ from donor

after 10h
→ from transconjugant

conjugative transfer occurred

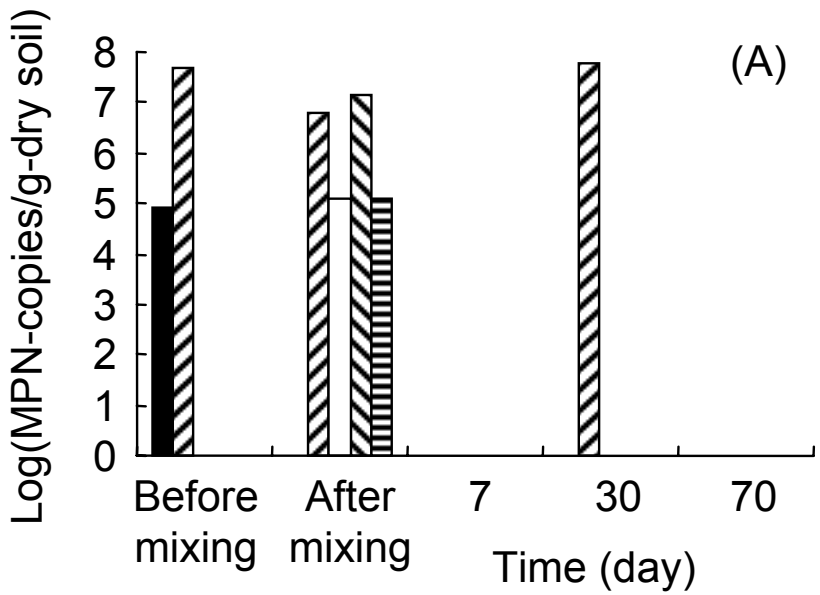
×:Donor, ○:Recipient, ●:Transconjugant
Real line from model calculation

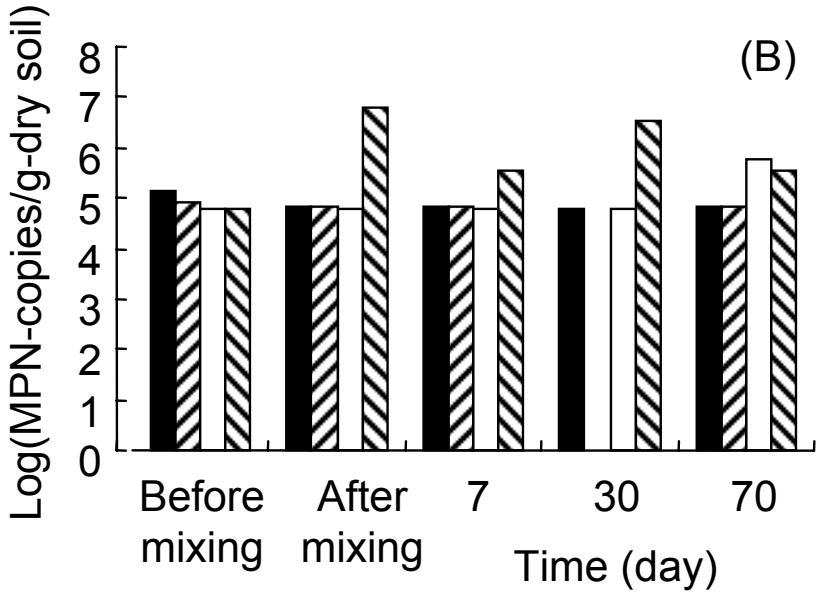
Estimation of the effect to microbial community

- Effect on element cycling function
- Effect on diversity of microbial community
- Estimation of the risk to indigenous microbial community

Bioremediation site of the oil polluted soil

Field experiment –Behavior of functional bacteria during bioremediation of oil polluted soil-





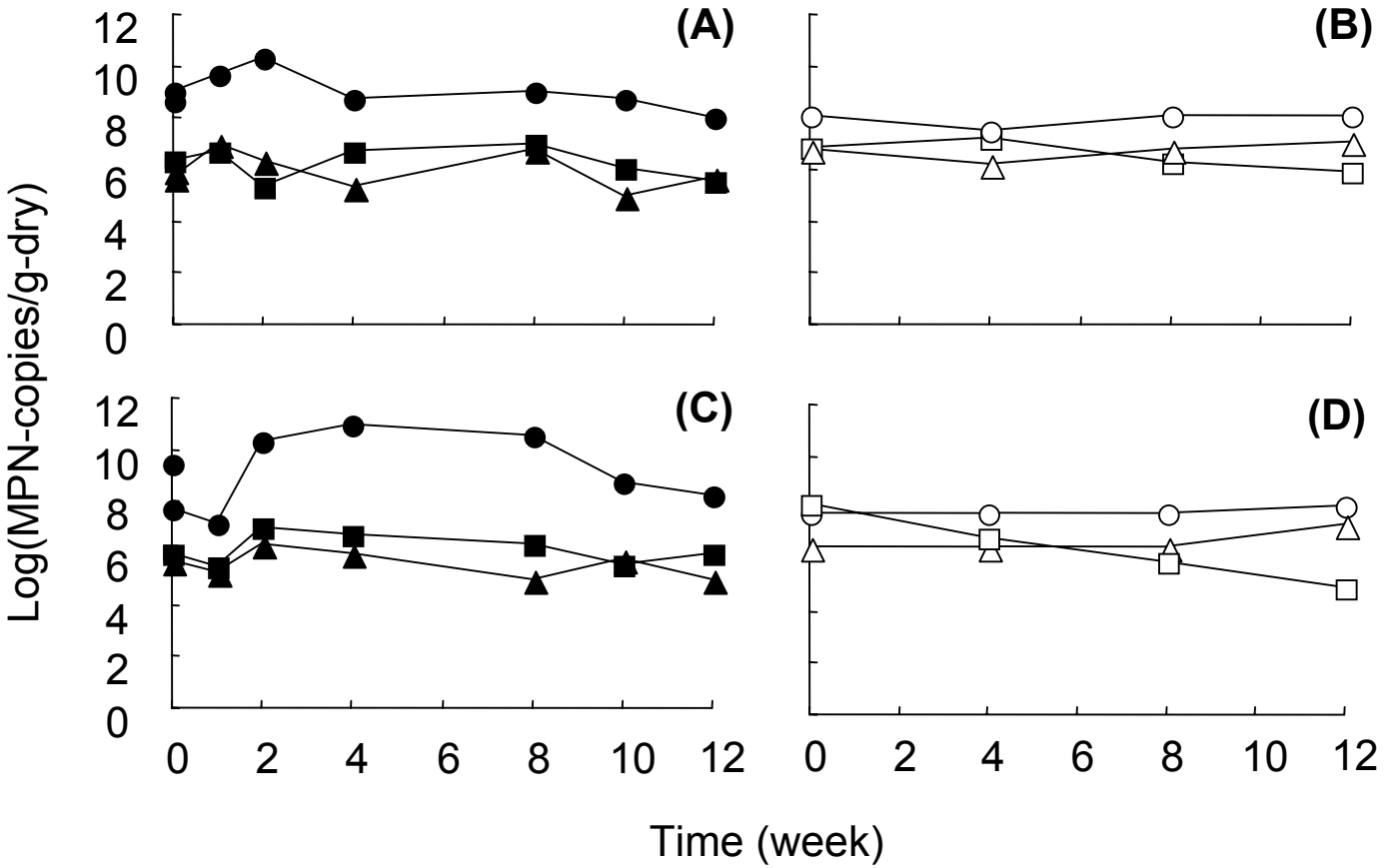
(A) Aromatic compounds and alkane degrading genes
Symbols; ■: C12O, ▨: C23O genes (Site 1), □: C12O, ▧: C23O, ▤: ALK3 genes (Site 2)

(B) Nitrous cycling genes
Symbols; ■: *amoA*, ▨: *nirK* genes (Site 1), □: *amoA*, ▧: *nirK* genes (Site 2)

Aromatic compounds and alkane degrading bacteria in introduced black dirt decreased under detection limit

Almost no effect on nitrous cycling bacteria

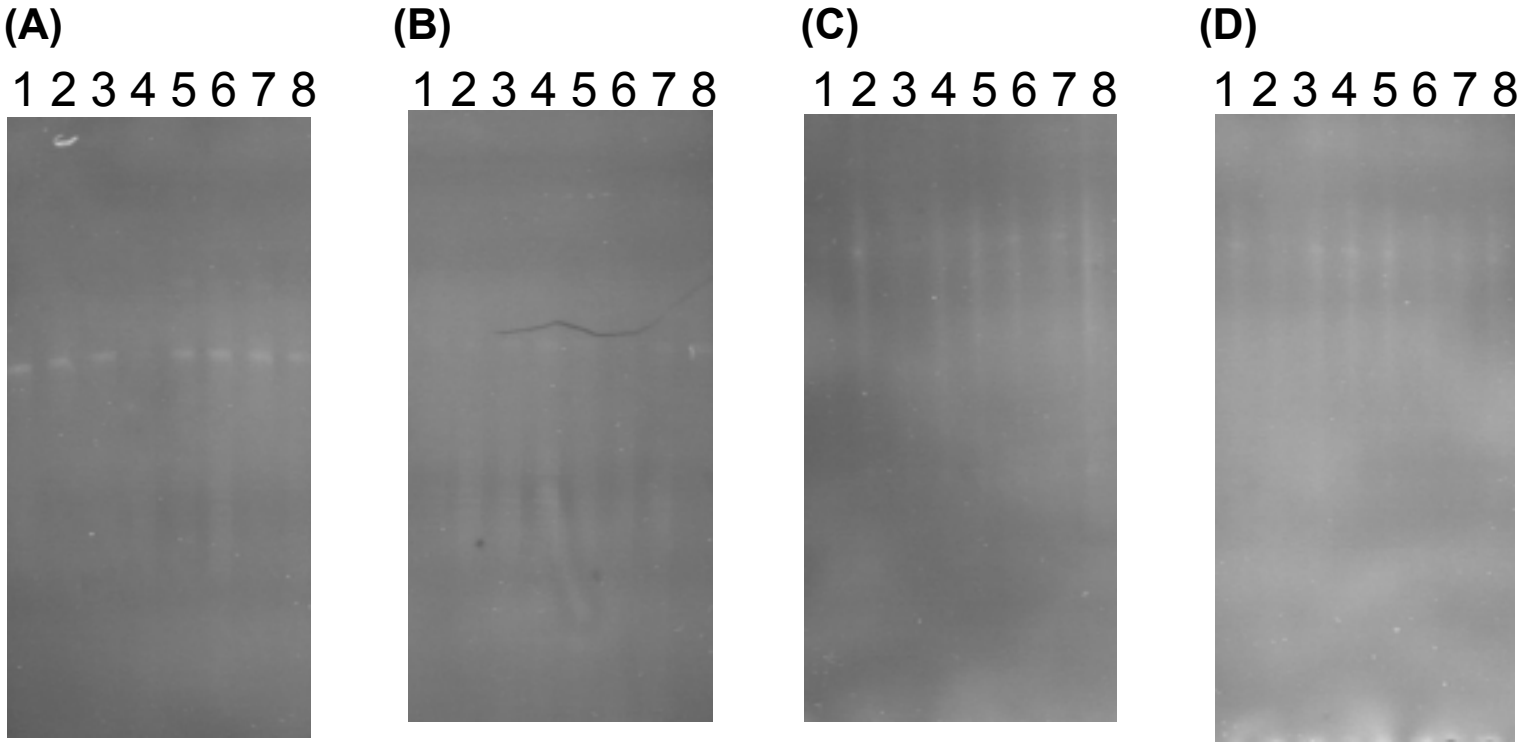
Field experiment –Behavior of functional bacteria during bioremediation of dioxin polluted soil-



(A) and (B) Site A, (C) and (D) Site C
Symbols: 16S rDNA (●), *amoA* (▲)、*nirK* (■) genes
ammonium oxidizing bacteria (○), nitrite oxidizing bacteria (△), denitrifying bacteria (□)

Increase of 16S rDNA and decrease of denitrifying bacteria in Site C

Field experiment –Change of microbial community structure during bioremediation of dioxin polluted soil-



Change of 16S rDNA DGGE banding pattern during bioremediation of dioxin polluted soil using white rot fungi

(A) Site A, (B) Site C, (C) Site G1, (D) Site G2
Lane 1, 0 w (before introducing white rot fungi; lane 2, 0 w (after introducing white rot fungi); lane 3, 1 w; lane 4, 2 w; lane 5, 4 w; lane 6, 8 w; lane 7, 10 w; lane 8, 12 w

No remarkable change was observed

Conclusion of experimental results

I. Understanding of inherent risk in indigenous bacteria:

Microarray was almost developed to monitor pathogenic and other bacteria

→ Pathogens 1012 species, Other bacteria 912 genera

II. Modeling and collecting parameters for risk assessment of environmental release of GEMs:

Horizontal gene transfer potential in soil environment is almost understood

→ About 1 % of soil bacteria are possible mobilizer

→ Gene receptive potential in soil bacteria is about 10^{-2}

→ Gene transfer is strongly affected by temperature, concentration and components of organic compounds

→ Possibility of conjugative transfer in soil environment is quite low

III. Accumulation of **data from actual bioremediation**

Accumulating data on the behavior and effects of GEMs and GEDS

→ Labo-scale: GEMs rapidly decreased, while GEDS remained
Model was developed for understanding their behavior

→ Field-scale: Introduced bacteria decreased under the detection limit, and almost no effect to microbial community

Further perspectives

- Development of model for risk estimation
- Accumulation of monitoring data from actual remediation site

Accumulation of many data, parameters for modeling and proper estimation of risk will encourage the safe, effective, and accurate bioremediation project using GEMs