Isolation and Characterization of 4- Chlorobenzoate and 4-Hydroxybenzoate Degrading Bacteria and Study of The change in Community Structure Of El Pond Sediment in Response to 4-Hydroxybenzoate Application

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Abstract

Five isolates from different environment have been isolated and identified those can utilize p-hydroxybenzoate as sole source of carbon. Three isolates were identified from iron horse park those can use p-chlorobenzoate as sole source of carbon. 16s rDNA sequencing suggest that all these bacteria are related to *Pseudomonas sp* and are very close to each other in phylogenetic tree. p-hydroxybenzoate contamination of El pond sediments at 1mM conc. has no effect on the community structure till 5 days. 10 days samples showed the replacement of initial community by p-hydroxybenzoate degrader as suggested by DGGE and T-RFLP analysis.

Introduction

Large amounts of man-made chlorinated organic chemicals have been introduced into the environment as agricultural pesticides and industrial agents. These compounds are classified among the most persistent and toxic group due to their chemical structures. Among them chlorobenzoate are released in the environment as intermediates during biodegradation of polychlorinated biphenyls and herbicides. Most bacteria which degrade biphenyls and polychlorinated biphenyls accumulate he corresponding chlorobenzoic acids and thus chlorobenzoate degradation can be the rate

limiting step in the biodegradation process of these biphenyl compounds(Yi et.al 2000).

4-Chlorobenzoate (4-CBA)is water soluble and has low toxicity, which enables many researchers to use it as the model compounds in studying the biodegradation of halogenated aromatic compounds. Organisms that have been reported to be capable of degrading 4-CBA belong to several genera, including Arthrobacter, Pseudomonas, Alcaligenes, and Nocardia 4-chlorobenzoate is metabolized via formation of 4-hydroxybenzoate (4-HBA) by dechlorination or via 4-Chlorocatechol by benzoate dioxygenation (Kim and Picardal, 2000).

Much of the previous studies on 4-CBA and 4-HBAdegrading bacteriaowever mainly focused on degradative enzymes and pathways, and thus little information available on the the phenotypic and phylogenetic diversity of degrading isolates.

The study of microbial diversity and community dynamics is rapidly growing in microbial ecology. Interest in this area has been catalyzed by the rapid advancement of molecular ecological methodologies. Through the use of culture-independent molecular techniques, new insight into the composition of uncultivated microbial communities has been gained. It is now becoming possible to define the cause of time dependent changes in the health of a stressed ecosystem on the basis of the structural composition of the ecosystem population.

Aims of the Project

1) To isolate bacteria from various ecosystems those can utilize 4-chlorobenzoate and 4-hydroxybenzoate as sole source of carbon.

2) To study the change in community structure of the El pond sediments in response to addition of 4-hydroxybenzoate.

Material and Methods

Isolation of 4-chlorobenzoate and 4-hydroxybenzoate degrader

Following sites were used for the samplings

Sites	Nature of sites
El pond	Salt water
Garbage beach	Salt water
Marsh	Fresh water
Soil	Soil

Samples from these sites were directly applied to agar plates for aerobic microbes on shake tubes for anaerobic microbes. 4-chlorobenzoate or 4-hydroxybenzoate was only source of carbon for the enrichment.

Media for aerobic and anaerobic enrichment were the same except sulfide solution was not added into aerobic medium.

Samples were directly plated on the agar plates or shake tubes. After every alternate day, the growth of microbial colonies was monitored and transferred on the fresh plate when needed. After three such transfer (and when it looked pure under the microscope), bacterial colonies were identified using 16s rDNA sequencing.

Medium for Sulfate-reducing Bacteria

(after F. Widdel)

In a Widdel flask (Fig. 1), combine the following salts in dist. H_2O and autoclave togeth

	Freshwater (g/l)	Saltwater (g/l)
Na_2SO_4	4.0	4.0
KH_2PO_4	0.2	0.2
NH_4Cl	0.25	0.25
NaCl	1.0	20.0
$MgCl_2 \cdot 6H_2O$	0.4	3.0
KCl	0.5	0.5
$CaCl_2 \cdot 2H_2O$	0.15	0.15

ing, cool hot medium under atmosphere of N_2 or N_2/CO_2 (80/20). Keep the screw ... for a few minutes to allow anaerobic gas to replace the air above the hot medium. ool to room temperature and add the following sterile stock solutions:

Trace element solution	1.0 ml/l
12 vitamin solution	1.0 ml/l
Vitamin B_{12} solution	1.0 ml/l
Bicarbonate solution	30.0 ml/l

84 g $NaHCO_3$/l, saturated with CO_2, filtersterilized or autoclaved in closed bottles under CO_2.

2 M HCl or 1 M Na_2CO_3 to adjust pH (7.0-7.3). Add an additional 1/6 the volume of the Na_2S solution as 2 M HCl to compensate for increase in pH due to Na_2S addition.

Sulfide solution 1.5 ml/l

240 g Na_2S x 9 H_2O/l, autoclave under N_2.

Mix all additions well with the medium.

Trace Elements Solution (1000x concentrated) (this is similar to "SL12")

Dist. H_2O	987 ml
EDTA	5200 mg
Adjust pH to 6.0 with NaOH	
Add the following:	
$FeSO_4 \cdot 7H_2O$	2100 mg
H_3BO_3	30 mg
$MnCl_2 \cdot 4H_2O$	100 mg
$CoCl_2 \cdot 6H_2O$	190 mg
$NiCl_2 \cdot 6H2O$	24 mg
$CuCl_2 \cdot 2H_2O$	2 mg
$ZnSO_4 \cdot 7H_2O$	144 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg
Sodium Vanadate	25 mg
SH_2O	6 mg
	8 mg

. ill to a final volume of 1 liter and filter sterilize

12-Vitamin Solution (1000x concentrated)

K-Phosphate buffer, 10mM, pH 7.2	100 ml
Riboflavin	10 mg
Thiamine·HCl	100 mg
L-Ascorbic acid	100 mg
D-Ca-pantothenate	100 mg
Folic acid	100 mg
Niacinamide	100 mg
Nicotinic acid	100 mg
4-Aminobenzoic acid	100 mg
Pyridoxine·HCl	100 mg
Lipoic acid	100 mg
NAD	100 mg
Thiamine pyrophosphate	100 mg

Titrate with NaOH until dissolved; Filter sterilize and freeze in 10 ml aliquots

Vitamin B_{12} Solution (1000x concentrated)

Water	100 ml
Cyanocobalamin	100 mg

Titrate with HCl until dissolved; filter sterilize and freeze in 10 ml aliquots

PCR Amplification of DNA

16s rDNA was amplified using primer of eubacteria. Primer set sequences were as follows: Eub8F: 5'- AGAGTTTGATCCTGGCTCAG—3' and Eub1492R: 5'-GGTTACCCTTGTTACGACTT-3'. The PCR thermal cycle profile was- The initial denaturation step of 95oC for 5 min, followed by 25 cycles, 95oC for 30 second,55oC for 30 second and 72oC for 1 min., The cycle was completed by a final extension of 772oC for 5 min. After the amplification, the DNA was run on 1.25% agrose gel. Materials were sent for sequencing.

The isolates were identified using ARB software.

Change in community structure

Sediment samples were collected from El pond. One set of triplicate samples were treated with 1mM conc. of p-hydroxybenzoate one for aerobic and another for anaerobic systems. Triplicate samples were kept without treatment as controls. DNA was extracted form all samples (referred as 0 day sample) using MoBio UltraClean Soil DNA isolation kit. The samples were retreated on 5 and 10 days. DNA was extracted again after each treatment.

PCR for DGGE. The primer used for PCR were DGGE341F(GM5F) (5'CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCT ACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3'). The cycling programme consisted of an initial denaturing step of 94oC for 5 min, followed by 10 cycles of denaturation at 95oC for 1 min, annealing at 66oC (decreasing in each cycle by 1oC) for one min and an elongation step of 72oC for 3min. I performed another 15 cycles of 95oC for 1min, 56oC for 1min, and elongation at 72oC for 3 min. Each run ended with a final elongation step of 72oC for 5 min.

DGGE was performed as described by Moesender et.al (1999) on Bio-Rad system.. A polyacrylmide gradient gel (from 30% to 70%) was prepaerd and gel was run for 5 hours at constant voltage of 200V. The gel was post stained with Gelstar. The bands appear in the gel was photographed.

PCR for T-RFLP.- The primers used for PCR were 8F-FAM and 1492R. The PCR condition was similar to bactrial sequence analysis as described earlier. The PCR product was digested with restriction enzyme Rsa1 with buffer C. incubation was done for 2 hours at 37oC. samples were sent for capillary electrophoresis.

RESULTS AND DISCUSSIONS

Five isolates were identified that can use p-hydroxybenzoate as sole source of carbon from different environments.

p-hydroxybenzoate degrader	**source**
Isolate 1	soil
Isolate 3	Marsh (FW)
Isolate 4	El pond(SW)
Isolate 6	Garbage beach(SW)
Isolate 7	Marsh(FW)

As shown in phylogenetic tree (Fig-1) isolate-1 is very close to *Comamonas sp*, while isolate-3 is between *Vibrio* and *Curacaobacter.* Isolate-4 is near to marine bacterium in the tree while isolate-6 is relatively very near to cyanobacteria. Isolate –7 is nearer to *Comamonas sp.*.

Three isolates were identified to use p-chlorobenzoate as sole source of carbon.

p-chlobenzoate degrader	**Source**
Isolate 12	Marsh(FW)
Isolate 13	Marsh(FW)
Isolate 14	Marsh(FW)

All these three isolate were obtained from the Marsh sample and in the phylogenetic tree they all are very near to *Pseudomonas sp.*

These results point towards diversity of microorganisms in the environment having similar physiological profile.

DGGE profile of community as shown in figure-2a,b,c, was not very informative. However, careful analysis may suggest that there was no apparent change in the microbial community after 5 days of treatment. However, 10 days samples showed appearance of thick band in all treated samples corresponding to band of El-pond isolate that used p-hydroxybenzoate as sole source of carbon.
T-RFLP of 0 and 5 days sample showed no change in community analysis as shown in figure- 3. this result somewhat support the DGGE profile. However, we cannot conclude anything very confidently form these two analyses. Certainly more systemic and long study is required for conclusion.

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References

Kim, S and Picarrdal, F.W. (2000) A novel bacterium that utilizes monochlorobiphenyls and 4-chlorobenzoate as growth substrates. FEMS Microbiol. Lett.185, 225-229.

Yi, H-R, Min, K-H, Kim, C-K and Ka J-K (2000). Phylogenetic and phenotypic diversity of 4-chlorobenzoate-degrading bacteria isolated from soils. FEMS Microbiol. Ecol. 31, 53-60

Moeseneder, M. M, Arrieta, J.M, Muyzer, G. Winter, C and Herndl, G.J.(1999). Optimization of terminal restriction fragment lenth

polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 65,(8), 3518-3525.

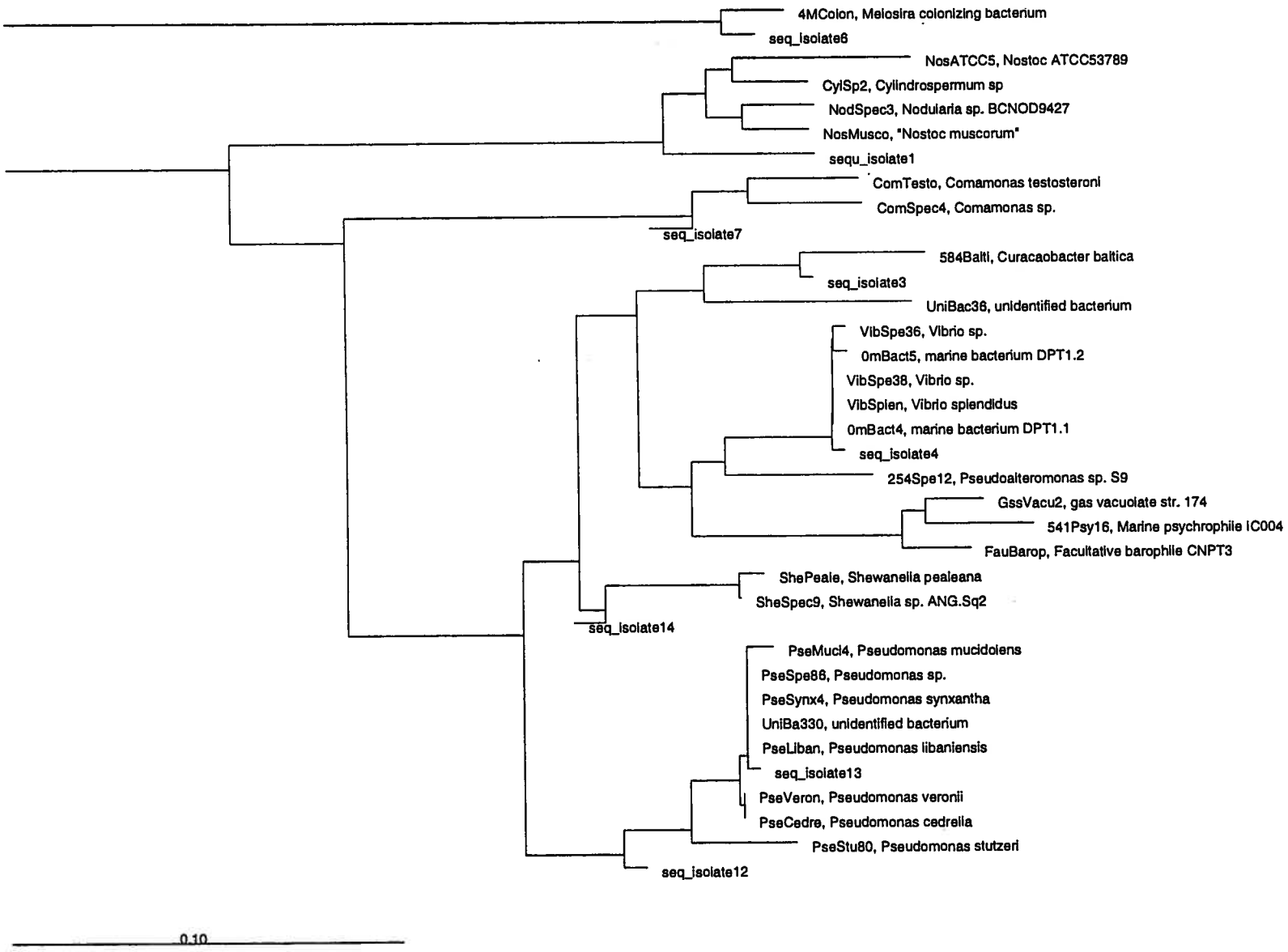


Fig-1 Phylogenetic tree for isolated bacteria.

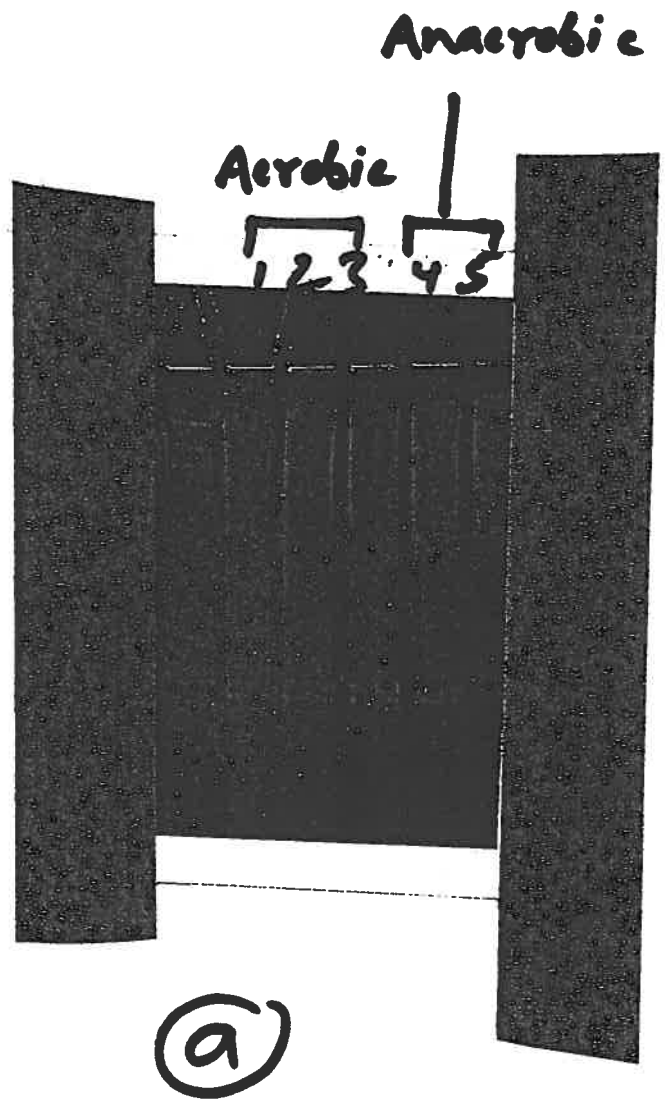
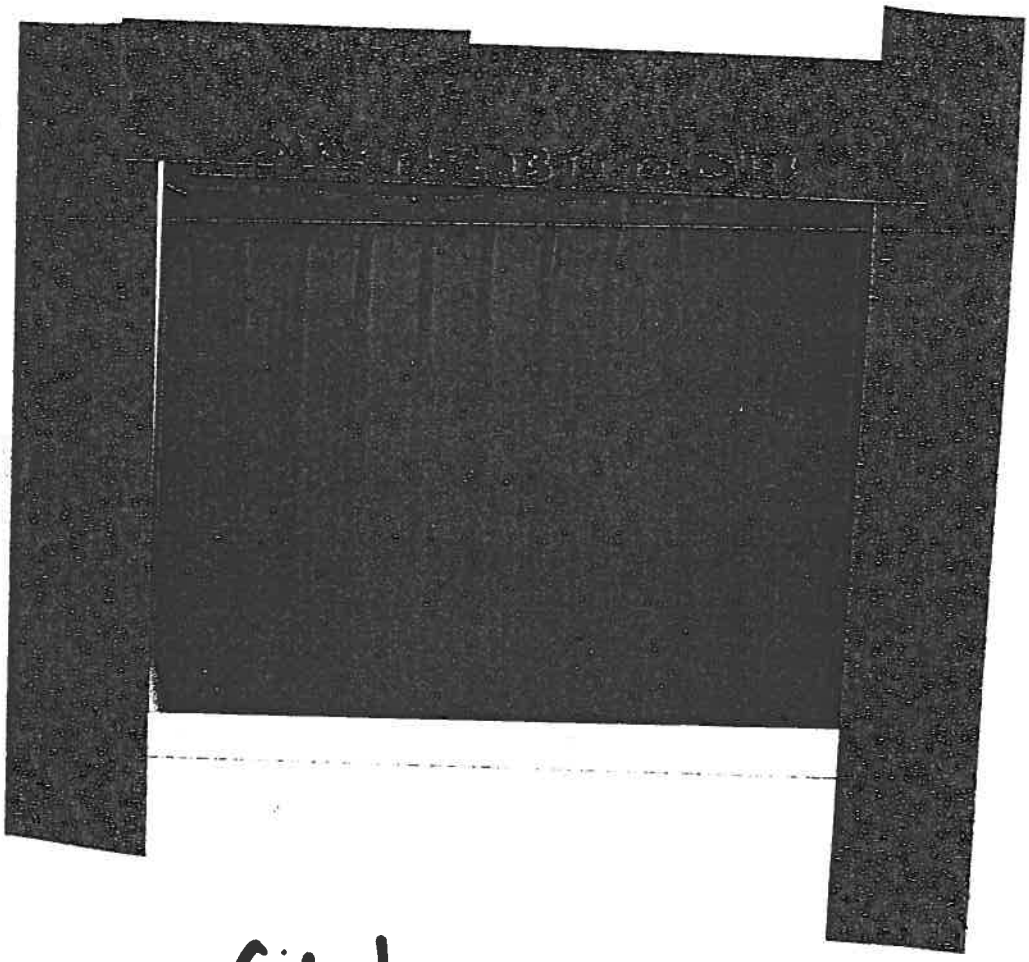


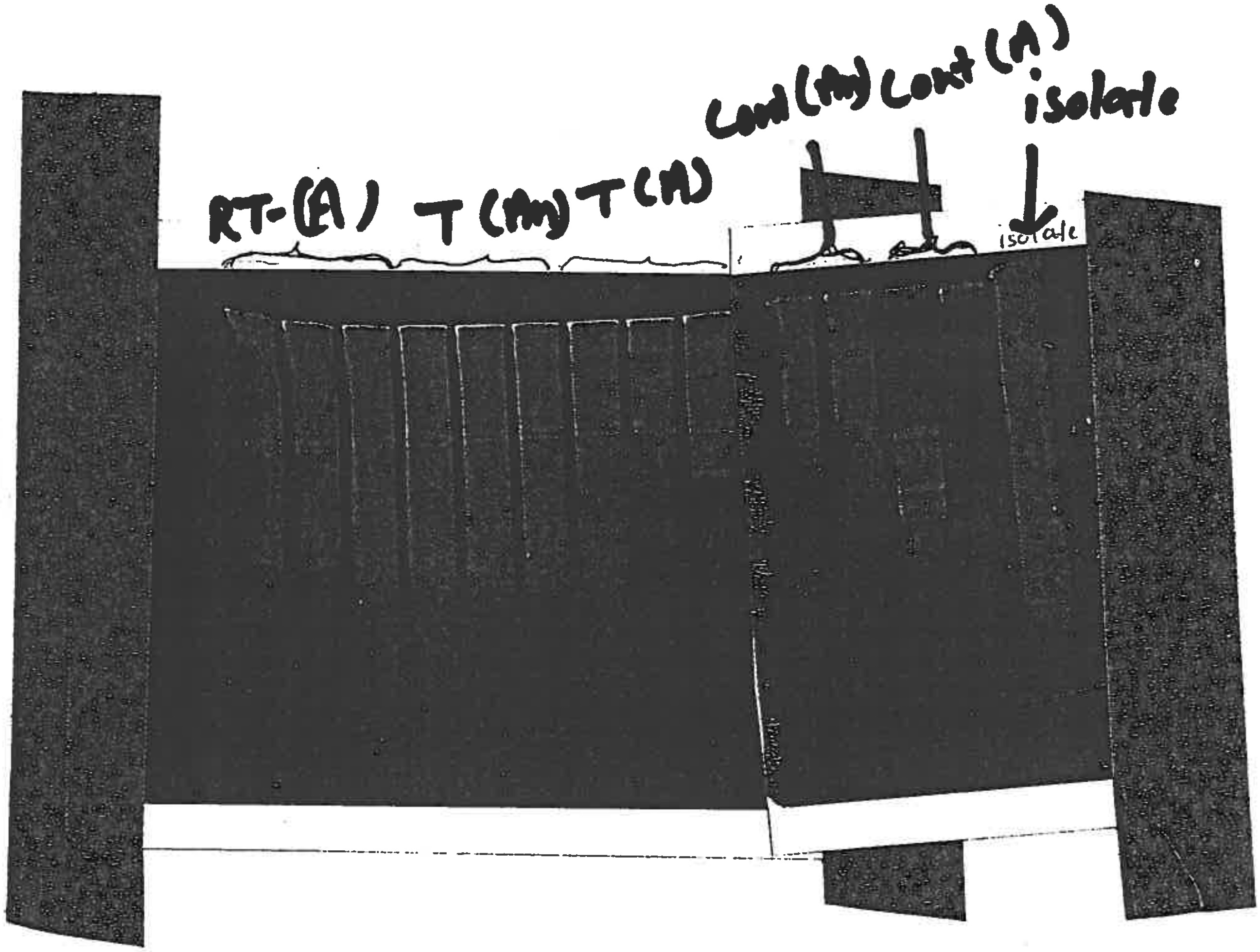
Fig-2 DGGE pattern from samples of different days a) 0 day sample, b) 5 days sample, 3) 10 days sample

5 Days Sample



(b)

10 Days Sample



(C)

10 Days

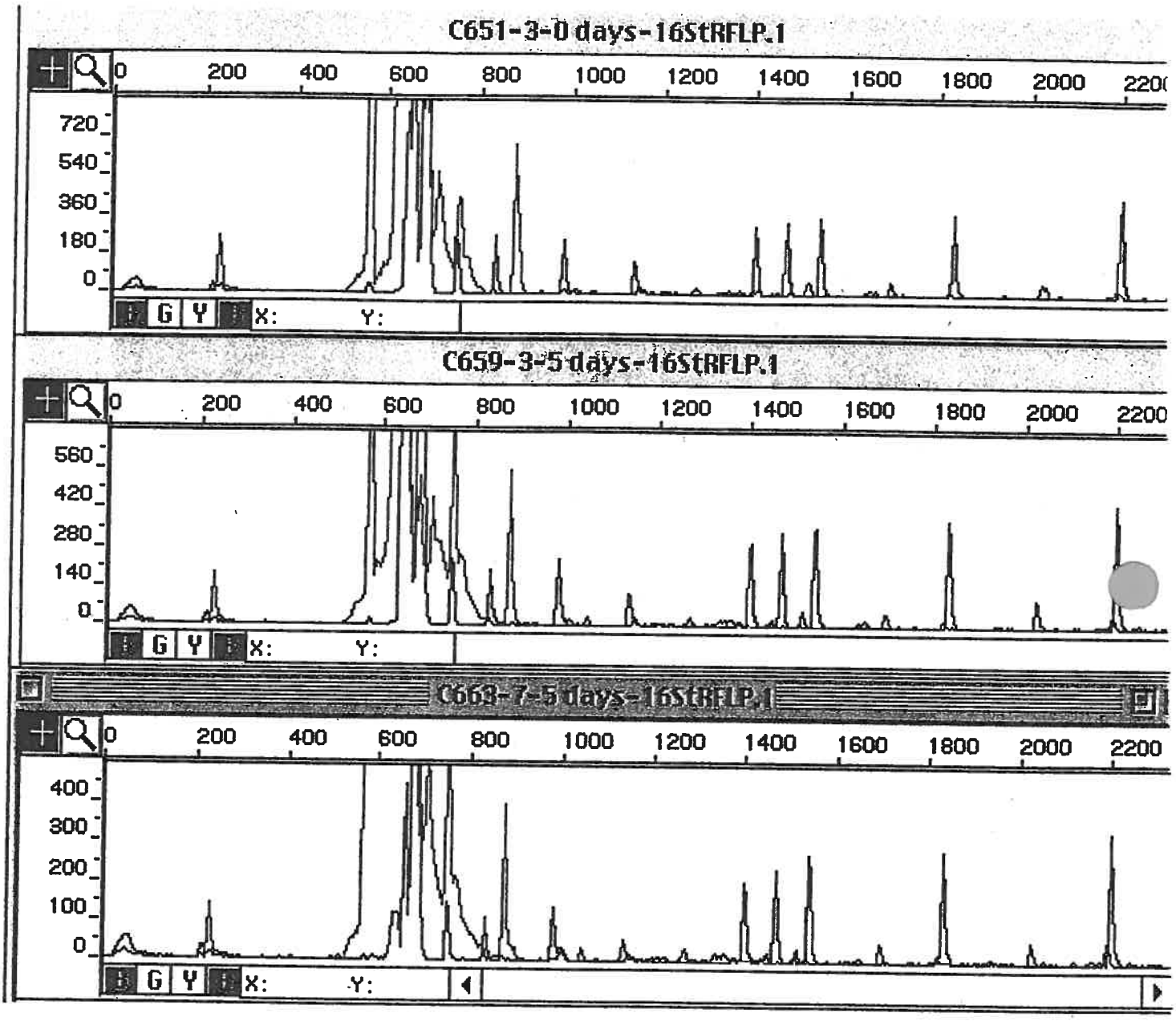


Fig-3 T-RFLP fingerprinting of marine bacterial community of El pond (0 and 5 days)