Co-metabolism of trichloroethylene by phenol grown aerobic granules

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Co-metabolism of Trichloroethylene by Phenol Grown Aerobic Granules

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ABSTRACT

This thesis focuses on the topic of co-metabolism of trichloroethylene (TCE) by aerobic granules fed on phenol. Extensive literature research and review were conducted in the areas of co-metabolic degradation of recalcitrant compounds, aerobic and anaerobic TCE degradation, phenol degradation and aerobic granular sludge. Experiments were designed and conducted in the following aspects, to explore the feasibility of sustainable TCE degradation operation by aerobic granular sludge and reactor.

Aerobic granulation process was carried out using phenol as the growth substrate. Changes of sludge characteristics and phenol/TCE degradation activities were monitored for four months until stable aerobic granules were formed. The stable granules retained most of the instant phenol and TCE degradation activity of the seed sludge, and lost half of the sludge's ultimate TCE transformation capacity.

Mature granules had the kinetic parameters of $V_{\max}=1.59$ mg phenol g SS⁻¹ min⁻¹, $K_S=37.2$ mg L⁻¹, $K_I=461.8$ mg L⁻¹ for phenol; and $V_{\max}=0.0142$ mg TCE g SS⁻¹ min⁻¹, $K_S=1.02$ g L⁻¹ for TCE. 1 g (dry weight) of stable granules could transform 1.1 mg TCE before the reaction stopped, and 2.8 mol chloride ions could be released per mol TCE transformed, indicating nearly total mineralization.

Exogenous reducing energy supply didn't have significant effect on the instant TCE degradation by the granules, but could enhance the ultimate transformation capacity by approximately 30%. Granular size was found to greatly affect phenol and TCE transformation. Bigger granules exhibited lower phenol and TCE degradation rate and capacity than smaller granules or flocs.

The toxicity of TCE and/or its transformation products were studied. Direct addition of TCE didn't have significant induction effect or solvent stress on the

granular sludge. The transformation products of TCE were found to reduce the subsequent phenol degradation activities of the granules, but only when larger quantities of TCE were transformed. Lower dosages of TCE could enhance subsequent phenol uptake, and such effect was sustainable and unique.

Isolation of pure phenol-degrading cultures was conducted, and six isolates were obtained. Together with a previous batch of pure cultures isolated from phenol granules, they were studied for their phenol and TCE transformation kinetics, TCE transformation capacities, growth yields on phenol and cell surface properties. Wide diversity was found with all the parameters studied, and several pure cultures were selected with potential of efficient TCE degradation.

A glass reactor was designed and constructed for the application of TCE co-metabolism by aerobic granules, with phenol as the primary substrate. The reactors were operated for six weeks, during which TCE and phenol were alternately fed. The reactors and the granular sludge inside were able to completely remove the fed TCE, by biological functions instead of adsorption or leakage. The structure of the aerobic granular sludge was maintained in the reactors where TCE was fed, but almost completely disintegrated in the control reactor without TCE feeding.

In conclusion, the aerobic granular sludge could efficiently co-metabolize TCE with phenol as the growth substrate, and showed some interesting characteristics, such as the response to TCE transformation product toxicity. Aerobic granular sludge reactors could be sustainably operated for TCE co-metabolism operation, and is a promising option for future application.

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LIST OF ABBREVIATIONS

2HMSH	2-hydroxymuconic semialdehyde hydroxylase
2HMSD	2-hydroxymuconic semialdehyde dehydrogenase
AGSB	aerobic granular sludge blanket
AMO	ammonia monooxygenase
BOD	biochemical oxygen demand
C12O	catechol 1,2-dioxygenase
C23O	catechol 2,3-dioxygenase
CAH	chlorinated aliphatic hydrocarbon
CF	chloroform
CO	carbon monoxide
CO ₂	carbon dioxide
Co-A	coenzyme A
COD	chemical oxygen demand
CT	carbon tetrachloride
DCE	dichloroethylene
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DW	dry weight
EPA	Environmental Protection Agency
EPS	extracellular polymeric substances
FID	flame ionization detector
HAO	hydroxylamine oxidoreductase
HPLC	high performance liquid chromatography
GC	gas chromatography
I.D.	inner diameter
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride
K _i	inhibition constant

K_s	half saturation constant
MMO	methane monooxygenase
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids
MP	mineral phosphate
NADH	reduced nicotinamide adenine dinucleotide
O.D.	outer diameter
OLR	organic loading rate
PCE	perchloroethylene
PH	phenol hydroxylase
PHA	polyhydroxyalkanoates
PHB	poly- β -hydroxybutyrate
pMMO	particle methane monooxygenase
REP-PCR	Repetitive extragenic palindromic sequence- polymerase chain reaction
S	concentration of the substrate
S^*	substrate concentration corresponding to the actual maximum transformation velocity
SBR	sequencing batch reactor
sMMO	soluble methane monooxygenase
SOUR	specific oxygen utilization rate
SS	suspended solids
SVI	sludge volume index
T2MO	toluene 2-monooxygenase
T3MO	toluene 3-monooxygenase
T4MO	toluene 4-monooxygenase
T_c	transformation capacity
TCA	trichloroethane
TCE	trichloroethylene
TDO	toluene 2,3-dioxygenase
T_e	transformation extent
TOUR	total oxygen utilization rate

TPT	transformation product toxicity
T_r	transformation rate
T_y	transformation yield
UASB	upflow anaerobic sludge blanket
V	specific transformation velocity
V^*	actual maximum transformation velocity
VC	vinyl chloride
$V_{\max}$	maximum specific transformation velocity
VSS	volatile suspended solids
y	growth yield
YEPG	yeast extract-pepton-glucose
Y_{obs}	observed growth yield

CHAPTER ONE

INTRODUCTION

1.1 Co-metabolism of recalcitrant compounds

Every year, natural and human activities produce and discharge million tones of substances to the environment. For a sustainable cycle of matters and balance of ecosystem, these substances often need to be transformed to other states, for instance, metals ions of certain value to another value, organic compounds to carbon dioxide (Allsopp et al., 2004).

Degradation is defined as the process where the complexity of chemicals is reduced (Alexander, 1999). An important aspect of degradation is the decomposition of carbon-containing organic matters to compounds of less carbon atoms and simpler structures. The degradation of organic compounds can be mediated by chemical and biological functions, aided by physical processes. Among the agents carrying out the degradation functions, microorganisms are of particular importance, because of their extremely small volume (hence high surface area to volume ratio), high metabolism rate, and the ability to carry out various reactions with high efficiency under ambient pressure and temperature (Singh and Ward, 2004).

When microorganisms degrade organic compounds, carbon atoms are taken off from the compounds' chemical structures. Some of the carbon atoms are assimilated by microorganisms to form cellular materials, and others could be oxidized or reduced to the extreme state, forming carbon dioxide or methane, respectively.

In some cases, energy can be derived from the chemical reactions in the degradation processes, while in the others, the microbes mainly rely on solar energy for living

(phototrophic). Microorganisms can also obtain substances from the biodegradation of chemicals to constitute their cellular structures, and this process is defined as growth-linked biodegradation (Alexander, 1999). The traditional biological treatment processes are depended on the growth of certain microorganisms on the target compounds, which can decrease the compounds' concentrations in the environment, detoxify the poisonous ones, release the carbon atoms, and facilitate the continuous cycle of matters (Tally, 2006). This process is self-sufficient and sustainable, as the microbes can “grow” on the substances and produce biomass to carry out the function.

However, some organic compounds, predominantly used in various industries, were invented by humans only recently. They are foreign to the nature, and their existence in the environment is not long enough for the local microbes to develop suitable degradation pathways for their destruction (Alexander, 1981; Sheehan, 1997). Therefore the microorganisms cannot grow on these substances, and they tend to accumulate in the environment, which could be very problematic when they are of acute or chronicle toxicity, and discharged in large quantities.

Trichloroethylene (TCE) is a good example of such a kind of chemicals. It is a chlorine-substituted short chain aliphatic hydrocarbon, existing in mobile liquid state under ambient conditions. Its good solvent property, high volatility and chemical stability, and greatly reduced potential for fire or explosion have enabled it to be intensively used as degreaser, dry cleaning agent and fumigants (Green, 1984). However, the analogs of TCE, e.g. vinyl chloride, dichloroethylenes have been identified as potent human carcinogens, and TCE itself is suspected too (Cohen, 1984). Therefore, the concentration of TCE in the groundwater used for drinking water supply is under strict regulation. For example, USA Environment Protection Agency has placed a limit of TCE concentration of $5 \mu\text{g L}^{-1}$ in the groundwater.

Large quantities of TCE are being produced and applied each year, which has resulted in the accumulation of TCE in the soil and aquifer, probably caused by

careless handling, spillage, leakage and illegal dumping. For instance, the annual production in USA only in year 1991 was 145,280 tons (<http://www.epa.gov/OGWDW/dwh/t-voc/trichlor.html>), and the TCE concentrations in some polluted sites could mount to the saturation concentration of 1084 mg L⁻¹ (Lowe et al., 2002).

The huge discrepancy between the regulated and actual TCE concentrations in the environment has called for designing of treatment processes. TCE could be removed by physical processes as air stripping etc., but it would only transfer TCE from one phase to another phase of the ecosystem, without destruction or detoxification. Chemical processes can be quite efficient in treating simple organic compounds as TCE, yet their advantages are compromised by the large quantities of polluted groundwater to be treated, and the relatively low TCE concentrations dissolved in it. As chemical treatment processes frequently require the pumping out of the polluted water (pump-and-treat), the efficiency is usually much lower than in-situ bioremediation (Rittmann et al., 1994).

As a consequence, biological processes have attracted the attentions of researchers and engineers in treating TCE contaminated water and soil. The most significant advantages of biological TCE treatment is its lower cost compared to chemical processes, and the ability to totally mineralize TCE to non-toxic end products (Chu & Alvarez-Cohen, 1996). In addition, biological processes can be more environmental-friendly, as the indigenous microbial populations are good candidates for cleaning up the polluted soil and groundwater, thus exerting the least environment impact on the ecosystem (Providerti et al., 1993; Ward et al., 2003).

The main problem of biological TCE treatment is that, TCE seems not to support microbial growth, i.e. TCE degradation either does not generate enough energy for the synthesis of cellular material, or cannot provide enough carbon atoms, or neither. Very few microorganisms have been recorded to “grow” on TCE and its kind as the sole carbon and energy source. *Dehalobacterium formicoaceticum* Strain DMC was the first pure culture isolated to grow on dichloromethane under

anaerobic condition (Mägli et al., 1996), and the effort of obtaining more is underway (Hata et al., 2004). Aerobic growth of pure bacterial cultures on less chlorinated compounds, e.g. vinyl chloride, dichloromethane or dichloroethylene has been observed (Oldenhuis et al., 1991; Dolfing et al., 1993), but no microorganism to-date has been found to grow on TCE.

Aerobic co-metabolism has received increasing interest since it was found for the first time that TCE could be degraded to carbon dioxide under aerobic conditions in soil columns supplied with natural gas (77% methane) (Wilson and Wilson, 1985). Co-metabolism is the mechanism of organic compound degradation where the target compound is degraded by microorganisms grown on other substances. During the growth, the microorganisms synthesize enzymes (biocatalyst) for the breaking down of the “primary” substrate, and gain energy and carbon. Some of the enzymes are not strictly designed for one substrate only, but can catalyze many other reactions, including the degradation of the target compound (Alexander, 1999).

The mechanism of co-metabolism can be illustrated in the following Figure 1.1.

Fig. 1.1. Mechanism of co-metabolism.

For example, methanotrophic bacteria can produce the enzyme of methane monooxygenase to convert methane into methanol, which is the first step of methane degradation (Alvarez-Cohen and McCarty, 1991c). Methane monooxygenase is an enzyme with broad specificity, and can catalyze many oxygenating reactions, one of which is the first step of TCE aerobic co-metabolism, TCE to TCE epoxide (Dalton & Stirling, 1982; Patel et al., 1982). Therefore, bacteria can be grown on methane as the primary and growth supporting substrate, then be used to degrade TCE as the secondary and co-substrate. Such a process is defined as TCE aerobic co-metabolism.

1.2 Aerobic co-metabolic degradation of trichloroethylene (TCE)

As mentioned above, TCE is a widely applied synthetic solvent, and a common pollutant in the groundwater (Storck, 1987). Due to its huge production, suspected carcinogenicity and potential of complete detoxification by aerobic degradation, TCE has been extensively studied as a model compound for the mechanism, limitation and application of aerobic co-metabolism. The findings made on TCE aerobic co-metabolism can serve as an indicator for the degradation of other similarly recalcitrant compounds, e.g. dichloroethylene isomers.

TCE can be degraded both anaerobically and aerobically. Under anaerobic conditions, only a very limited variety of bacteria have been found to degrade TCE and grow on the process, others are postulated to reductively dechlorinate TCE as the ultimate electron acceptor only or via a co-metabolic mechanism (McCarty, 1997; Fantroussi et al., 1998). In an anaerobic cometabolic process, it is suggested that a part of the electrons generated in the degradation processes of other compounds are diverted to TCE, and TCE is reduced to less chlorinated substances.

The anaerobic TCE degradation by bacteria growing on the process has not been studied extensively, its mechanism is not thoroughly understood, and its application is still limited. For the co-metabolic anaerobic degradation of TCE, a problem exists that, the products from TCE reduction are stepwisely dichloroethylene (DCE)

isomers, vinyl chloride (VC), ethylene and ethane, which have decreasing chlorine contents. The less chloride content a compound has, the lower is its potential for reductive degradation (Vogel, 1987). Therefore the end products, ethylene and ethane are generated with relatively low rate, and DCEs and VC can be accumulated. These compounds are notorious human carcinogens, and should be avoided in a treatment process.

Aerobic TCE degradation, in contrast, can completely mineralize TCE to the non-toxic end products of water, carbon dioxide and chloride ion, therefore poses much less hazard on the environment compared to anaerobic dechlorination. No microorganism to date has been recorded to grow on TCE aerobically. All the degradation reactions occur via co-metabolic pathways.

In addition to methane, many other substances are able to support TCE co-metabolism, eg. ammonia, propane, isoprene, toluene, phenol, and cumene. The non-specific enzymes include ammonia monooxygenase, toluene mono- and dioxygenase, phenol hydroxylase and others. Among the primary substrates for TCE aerobic co-metabolism, aromatic compounds have been found to produce robust biomass that has high TCE removal efficiency (Folsom et al., 1990; Hopkins et al., 1993b). Within the aromatic compounds category, phenol is preferred than toluene, because of its lower toxicity to microorganisms and higher solubility (Fries et al., 1997).

Up to now, relatively few studies have been conducted on TCE co-metabolism by phenol degraders. Toluene has been used by most of the researchers as a representative of aromatic compounds. Toluene degraders, e.g. *Burkholderia cepacia* G4 exhibited outstanding TCE co-metabolic abilities (Newman & Wackett, 1995). However, the application of toluene is limited by its high toxicity to humans and microorganisms, and its low solubility increases the incident of evaporation and leakage. Phenol is an aromatic compound of similar structure to toluene, but is more soluble in water and less toxic. In addition, the above mentioned toluene degraders can also be grown on phenol as the primary substrate, and the enzyme

synthesized for TCE co-metabolism is not compromised for its activity (Spain et al., 1989). Therefore, phenol is selected as the growth substrate in this study for co-metabolic degradation of TCE.

Many pure and mixed cultures of bacteria have been characterized for their ability of TCE co-metabolism, e.g. methanotrophic, ammonia oxidizing, propane and toluene grown mixed cultures; ammonia oxidizing *Nitrosomonas europaea*, methane-grown *Methylosinus trichosporium* OB3b, and toluene degraders *Pseudomonas putida* F1 and *Ralstonia pickettii* PKO1. Pure cultures usually have higher TCE degradation rate and transformation capacity, but the application of pure cultures in real treatment processes is limited. One reason for the limitation is the high cost needed to maintain the purity of the culture, where all the reactor parts, feeding nutrient solutions and probably the pollutants to be treated require sterilization. Another reason is the survival rate of the pure cultures when they are applied for in situ bioaugmentation, where they have to compete with indigenous bacterial population already adapted to the local environment. If the pure cultures survive, the biosafety becomes another concern that the ecological impact of adding pure bacteria strains to the natural environment might be difficult to predict.

In this study, a special form of mixed bacteria culture will be applied in the study of aerobic TCE co-metabolism. Aerobic granular sludge has for the last ten years attracted increasing interest of researchers from all over the world. It is a kind of autoaggregate of mixed microbial populations under aerobic conditions (Tay et al., 2001a). Aerobic granules have many advantages in wastewater treatments processes. It has a compact structure and high density, enabling it to settle very fast within a reactor. The good settling ability further facilitates the retention of large quantity of biomass within the reactor, increasing the treatment efficiency of the process. As a result, the aerobic granular sludge reactor can have small foot print, short hydraulic retention time and high loading rate. Its high biomass retention is also advantageous to the treatment of recalcitrant compounds, because of the higher biomass/substrate ratio compared to common aerobic activated sludge processes.

Another advantage of aerobic granular sludge reactor is that the compact structure provides shelter for diverse microorganism populations and protection from high concentrations of toxic chemicals. The former enables the granular sludge to treat wastewater of various compositions, and quickly adapt when the composition changes. The latter is particular advantageous for the treatment of toxic compounds, e.g. phenol, as the multi-layer structure can create diffusion barrier to the inner cells in the granules, protecting them from high phenol concentrations. Aerobic granules have been successfully cultivated with phenol as the sole carbon and energy source (growth substrate), and have shown outstanding treatment efficiency and remarkable resistance to the toxicity (Jiang et al., 2002).

1.3 Objectives and scope

As the aerobic granular sludge has the above mentioned advantages in treating toxic and recalcitrant compounds, it is intended in this study to explore its possible application in the co-metabolic degradation of TCE, using phenol as the growth substrate. The treatment target of this aerobic granular co-metabolic process will possibly be wastewater stream or combination of wastewaters containing both phenol and TCE or TCE-kind substances. The TCE concentration to be treated will likely to be determined by the kinetic and toxicity parameters exhibited by the granules, but probably will be within the range of 1 to 20 mg L⁻¹. It would be the first time that aerobic granules are applied in TCE co-metabolism, and the first time a second substrate is used in aerobic granular sludge reactor, especially that the substrate is chlorinated and potentially toxic. The results obtained in this study can hopefully add new knowledge to the area of chlorinated hydrocarbon co-metabolism, and further the understanding on the function and applicability of aerobic granules.

It was intended that this study should answer the following questions:

- What is the difference between the phenol and TCE degradation behavior of activated sludge and aerobic granules?
- How do aerobic granules degrade TCE as a whole?
- How do the bacteria in the granules degrade TCE as individual pure cultures?
- How do aerobic granules degrade TCE in a reactor where phenol is fed as the primary substrate?

To answer these questions, the following aspects of TCE co-metabolism by phenol-grown aerobic granules were to be studied:

- formation of the granules on phenol
- change of phenol and TCE degradation behavior of the sludge during granulation
- the degradation kinetics of phenol and TCE by stable granules
- the toxic effect of phenol and TCE
- the pure cultures in the granules and their TCE/phenol degradation abilities
- construction of a phenol/TCE alternative degradation reactor

The aim is to explore the possibility of treating TCE containing wastewaters by phenol-grown aerobic granules in a sustainable manner.

1.4 Organization of the thesis

The thesis contains seven chapters. Chapter One is the introductory chapter, where the background, the basic concepts, and the importance of the study are briefly stated. The research of the literature is then conducted, and the results are summarized in Chapter Two, Literature Review. Three main aspects are covered in this chapter, the specific terms and mechanism of co-metabolism, the degradation of phenol, and the formation and characteristics of aerobic granules. Chapter Three

to Six contain the detailed research results of the study, following the logic sequence introduced in section 1.3. They are, Chapter Three, Granule formation on phenol and change of phenol/TCE degradation activity; Chapter Four, TCE co-metabolism activity by stable aerobic granules; Chapter Five, Isolation of phenol-degrading pure cultures from the granules and characterization of their TCE co-metabolic activity; and Chapter Six, Alternative phenol and TCE degradation in an aerobic granular sludge reactor. The main findings made in these four chapters are concluded in Chapter Seven, where suggestions on possible future directions of the study are also covered. Reference related in the main text follows Chapter Seven, at the end of the thesis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

In this chapter, the basic concepts that would be used this thesis are introduced, which include the backgrounds, mechanisms and terms commonly used in the area of co-metabolism, TCE degradation, phenol degradation and aerobic granules.

2.2 Co-metabolism

Most organic chemicals that can be found in nature are biodegradable. Microorganisms can biologically transform those organic compounds to substances containing less carbon, or completely break them down to inorganic end products. The chemicals being transformed are therefore “degraded”, and as the result, the microorganisms can obtain energy and grow.

However, with the development of science and industry, many new non-biodegradable chemicals are being produced and utilized. When these xenobiotic chemicals enter the natural environment, the degradation might become a problem. Because they are strange to the native microbial populations, the microorganisms cannot recognize and degrade them. The man-made substances then may be left intact for years or decades in the environment. As many synthetic chemicals are toxic or harmful to humans, animals and natural environment, this has become a threat to public health and eco-system.

Although the xenobiotic organic chemicals may not be directly biodegradable, they might be transformed by microorganisms in a co-metabolic way. Cells produce enzymes, a kind of biological catalysts, for the breaking down of the chemicals on which they grow. Different enzymes have different specificities. Some are very

specific and only function on a given substrate. Others are less “particular” and might catalyze a group of substances with or without similar chemical structures. Co-metabolism is conducted by enzymes of broad specificities. It is defined as the simultaneous metabolism of two compounds, in which the degradation of the second compound depends on the presence of the first one. In many cases, it means the fortuitous biotransformation of a non-growth-supporting compound by a microorganism. That is, the enzymes required in the degradation of the growth substrate cannot discriminate the foreign compound, and break it down as an “accident”.

2.2.1 Core constituents of co-metabolism

Fig. 2.1. The diagram of interrelationship of the core constituents in co-metabolism.

As shown above in Fig. 2.1, different from conventional two-factor systems (substrate and microorganisms) for organic compound degradation, there are three core constituents in the mechanism of co-metabolism: co-substrate, growth

substrate and biotic factor—microorganisms or the relative enzyme systems. There are profound interactions between and among the factors.

2.2.2 Primary substrate

The primary substrate is the physiological substrate of microorganisms. Microorganisms obtain energy and carbon for growth when they degrade the primary substrate, therefore it is also sometimes termed as “growth substrate”. Generally a primary substrate is necessary in co-metabolism in two ways. First, it is needed for the induction of the non-specific enzymes, e.g. methane for methane monooxygenase. Second, its degradation yields energy and carbon for cell maintenance and growth.

2.2.3 Co-substrate

Co-substrates are mostly xenobiotic substances, whose existences in nature are not long enough for the indigenous microorganisms to develop suitable enzyme systems to degrade them (Dolfing, 1993). The degradation of some of these substances can be catalyzed by enzymes originally designed for other compounds (primary substrate), but of a broad specificity. In other words, microorganisms do not use the co-substrates as the sole carbon and energy source, but degrade them fortuitously as a “mistake” (Horvath, 1972). The co-substrate is the target compound in a co-metabolic degradation.

2.2.4 Competitive inhibition between the primary and co-substrate

The primary substrates and co-substrates are essentially catalyzed by the same enzymes. When both substrates are present, they will compete for the binding to the relative enzyme active sites, therefore competitive inhibition of each other's degradation may occur (Bailey & Ollis, 1986). The existence of co-substrate is known to decrease the degradation rate of the primary substrate, and vice versa (Folsom et al., 1990; Alvarez-Cohen & McCarty, 1991b).

2.2.5 Methods to avoid competitive inhibition

Competitive inhibition is not desirable in the co-metabolism as it may negatively affect the degradation both by directly decreasing the degradation rate of the co-substrate, and by inhibiting the metabolism of the primary substrate, and hence retarding the microbial growth. To circumvent the competitive inhibition, a resting cells system was proposed (Alvarez-Cohen & McCarty, 1991a), where the primary substance was eliminated from the degradation process after the induction of enzymes. This was based on the fact that, after being inducted by the primary substrate, microorganisms could continue to degrade the co-substrate for a considerable period even if the primary substrate was depleted (Hou et al., 1984).

2.2.6 Disadvantage of resting cell systems

Complete resting cells are not sustainable in co-substrate degradation. The rate of such degradation will decrease and approach zero after a period of time (Oldenhuis et al., 1989; Tsien et al., 1989). A possible reason of this phenomenon is the depletion of reducing energy (Chang & Alvarez-Cohen, 1995a).

Reducing energy exists in the form of NAD(P)H (reduced nicotinamide adenine dinucleotide), which is needed in the oxidative steps of metabolism catalyzed by monooxygenases or dioxygenases. There are three sources of reducing energy (Arp, 1995):

- ❖ subsequent degradation of the growth substrates
- ❖ non-competitive substrates : oxidized by bacterium without involving the enzyme causing competition; can be the metabolic intermediates of the growth substrate
- ❖ endogenous storage compounds : poly- β -hydroxybutyrate(PHB), etc.

In the aerobic degradation of the natural substrates by the microorganisms (i.e. primary substrates), the reducing energy consumed in the beginning of oxidation reactions will be regenerated in the subsequent degradation steps. Thus the balance

of NADH will be achieved after the degradation process is completed, and the reaction is sustainable. But the degradation of co-substrates probably does not have such a character (Dalton & Stirling, 1982; Henry & Grbić-Galić, 1991b). NADH is believed not to be replenished in TCE degradation, or not directly available to the microorganisms oxidizing TCE. In such a case, the co-metabolism process will exert a net drain on the reducing energy pool in cells, causing the reaction to stop. This hypothesis has been verified in numerous experiments where the addition of a non-competitive, exogenous NADH source in co-metabolic systems enhanced the degradation of co-substrates (e.g. TCE) (Alvarez-Cohen & McCarty, 1991c), or the content of an endogenous reducing source such as PHB (poly- β -hydroxybutyrate) was manifested to increase the degradation rate in resting cells system (Henrysson & McCarty, 1993).

Other explanations of the unsustainable co-metabolism in resting cell systems are the loss of enzyme expression in microorganisms after a long period of starvation (Dalton et al., 1984) and the co-substrate toxicity (Mars et al., 1998).

2.2.7 Growing cells system

The opposite of resting cells are growing cells, which degrade the co-substrate in the presence of the primary substrate. Competitive inhibition then may be the main problem in such systems. To minimize the negative effect of competitive inhibition, the concentrations of both primary substrate and co-substrate should be carefully selected, taking into consideration the kinetic parameters exhibited by the degrading cultures for both substrates.

2.3 Trichloroethylene (TCE)

TCE is a widely used synthetic organic compound for industrial extraction, dry cleaning, degreasing and heat transfer (Green, 1984).

The application of TCE and other chlorinated aliphatic hydrocarbons has been prevalent in many countries. These chemicals are favored for the chemical stability, reduced potential for fire or explosion and excellent solvent property (Ensley, 1991). Due to the xenobiotic nature and stable chemical structure of chlorinated substances, the degradations are slow whether via chemical or biological pathways (Vogel, 1987). Meanwhile, these compounds are highly volatile, and many of them are proven or suspected human carcinogens (Cohen, 1984).

In USA, the annual production of trichloroethylene has increased from just over 118 tons in 1981 to 145,280 tons in 1991. Due to careless handling, uninformed disposal, and landfill leakage, the amounts released to water and land are over 45 tons and 87 tons, respectively from 1987 to 1993 (<http://www.epa.gov/OGWDW/dwh/t-voc/trichlor.html>). As a consequence, TCE is listed as the most commonly reported volatile organic pollutant of groundwater in the United States.

Different pollution levels of TCE in the ground waters and soil systems have been reported. A typical pollution level of 0.5 mg L^{-1} with the range of $0.002\sim 1.2 \text{ mg L}^{-1}$ was reported by Hanada in the soil and ground water samples from a contaminated site in Japan (Hanada et al., 1998). In Denmark, a much higher concentration (9 mg L^{-1}) was observed in groundwater near two contaminated sites (Broholm et al., 1990). In USA., pollution levels may range from 10 to 100 of $\mu\text{g L}^{-1}$ in contaminated groundwaters (Westrick, 1990), while even saturation concentration of TCE (1084 mg L^{-1}) was found in a Superfund site (Lowe et al., 2002)

In contrast, the regulated maximum contamination level of TCE in groundwaters is $5 \mu\text{g L}^{-1}$ by US EPA. The huge discrepancy between the pollution level and the

regulation level, and the potential hazard of TCE emphasize on the necessity of its elimination from the natural environment.

Various physical and chemical methods have been used to clean up the contaminated groundwater system of chlorinated hydrocarbon pollution, such as air stripping, incineration, activated carbon sorption, solvent extraction and chemical catalytic oxidation. But conventional physical and chemical treatments suffer from high cost (Fujita et al., 1995) or low efficiency, for they only transfer these pollutants from one phase to another without completely destroying their structures (Alvarez-Cohen & McCarty, 1991d). Biological degradation then has been proposed as a promising alternative (Chu & Alvarez-Cohen, 1996).

2.3.1 Structure of TCE

Fig. 2.2. The molecular structure of TCE.

The chemical structure of TCE is shown in Fig. 2.2. The backbone of TCE is ethylene, with three hydrogen atoms substituted by chlorine atoms (Merck Index).

2.3.2 Physical property

- ❖ Good solvent property
- ❖ High volatility: dimensionless Henry's law constant: 0.4 at 25 °C
- ❖ Non-flammable mobile liquid, with the density of 1.45 g cm⁻³ at 25 °C
- ❖ Solubility in water: 1100 mg L⁻¹ at 25°C
- ❖ log K_{OW} : 2.61

2.2.3 Chemical property

- ❖ Stable
- ❖ Slightly polar
- ❖ Highly oxidized
- ❖ Slowly decomposes by light in moisture with the formation of HCl

2.3.4 Degradation of TCE

TCE belongs to the family of chlorinated aliphatic hydrocarbons (CAHs). The most frequently applied short chain chlorinated hydrocarbons in industries and scientific researches include: CF (chloroform), CT (carbon tetrachloride), 1,1,1-TCA (trichloroethane), TCE, VC (vinyl chloride), DCEs (1,1-, cis-and trans-1,2-dichloroethylene) and PCE (tetrachloroethylene).

The biodegradability of CAHs is much related to their structures and numbers of chloride substitutes. The higher the degree of chlorination of a compound, the lower is its rate of oxidative degradation, the more susceptible it is to reduction.

2.3.4.1 Photolysis & chemical transformation

TCE does not adsorb light of less than 290 nm and therefore could not be directly photo-degraded. However, slow (half-life 10.7 months) photo-oxidation in water has been noted (<http://www.epa.gov/OGWDW/dwh/t-voc/trichlor.html>).

Chemical transformation of halogenated compounds in aqueous solution, such as substitution and dehydrohalogenation in the absence of inorganic or biochemical catalysts usually proceed slowly. The half-lives of polychlorinated species are usually on the scale of years, e.g. the half-life of TCE by abiotic hydrolysis or dehydrohalogenation at 20°C is 0.9 year (Dilling et al., 1975).

2.3.4.2 Biotic transformation in mammalian system

Degradations of chlorinated hydrocarbons in abiotic, mammalian and microbial systems share similar reaction mechanisms and transformation products (Vogel, 1987; Ensley, 1991). Among them, biologically mediated oxidation of organic compounds has been extensively studied. These reactions are mostly catalyzed by monooxygenases that contains cytochrome P450, a heme-containing protein with iron-porphyrin active sites that can mediate both oxidation and reduction.

2.3.4.3 TCE epoxidation

The epoxidation of TCE is a well documented mechanism in mammalian systems (Miller, 1983). It's also proposed as the most important and rate-limiting step of halogenated alkenes oxidation in aerobic microorganisms (Semprini, 1997). The double bond in TCE is oxidized, with the formation of epoxide, an unstable intermediate, which may undergo several different chemical or biological reactions subsequently.

Fig. 2.3. Fate of TCE epoxide in aqueous solution without catalysis (Vogel, 1987).

In aqueous solutions, TCE epoxide is very unstable and may undergo spontaneous degradation (van Hylckama Vlieg et al., 1997). The reaction type and products are much dependent on the pH of the solution, as shown in Fig. 2.3. Under neutral condition, the main product is dichloroacetic acid. In acid environment, glyoxylate dominates. At a slightly higher pH, the epoxide decomposes to formyl chloride and dichloromethanol; the former eliminates HCl to form carbon monoxide; and the latter hydrolyzes to form formate (Henschler et al., 1979). TCE epoxide is a short-lived substance, with a half life of only 10~20 s (Fox et al., 1990; van Hylckama Vlieg et al., 1996).

2.3.4.4 Biotic degradation by microorganisms

Both aerobic and anaerobic degradations of TCE may occur in the natural environment.

Theoretically, reductive degradation is favored for TCE, because TCE has three chlorine substitutes, and therefore is in a relatively high oxidation state. However, only a limited number of microorganisms have been found to use TCE as the sole carbon and energy source under anaerobic conditions (Fantroussi et al., 1998; Holliger et al., 1999). Others are proposed to use TCE as an electron acceptor but not a carbon source, or via a completely fortuitous co-metabolic mechanism. The mechanism of reductive dechlorination under anaerobic or anoxic conditions is not yet well understood.

In the limited studies with undefined biological systems or defined microbial consortia and pure cultures, it was discovered that reductive dechlorination of TCE can be conducted under methanogenic (Freedman & Gosset, 1989), denitrifying, SO_4^- respiration, iron reducing and fermenting conditions (Cobb & Bouwer, 1991; Boopathy & Peters, 2001). It was proposed that a portion of electrons could be diverted from the normal reaction procedure to the reductive dechlorination process, therefore highly chlorinated substances, e.g. TCE or PCE were degraded to less chlorinated DCEs or VC.

The concern in using anaerobic degradation pathways to bioremediate TCE polluted groundwater is the possible formation of toxic intermediates or end-products. Theoretically, the degradation pathway in anoxic underground environment may proceed as follows (Vogel & McCarty, 1985).

Ethane was also observed as the end product of anaerobic dechlorination (de Bruin et al., 1992). Then ethylene or ethane is metabolized through conventional aerobic degradation pathway to form CO₂ (Dolfing et al., 1993). But studies have shown that this pathway may not be conducted straight to the end by anaerobic microbes, because the final dechlorination step (VC to ethylene) appears to be rate limiting (DiStefano et al., 1991). Therefore the degradation may result in the accumulation of DCE or VC, without the necessary occurrence of complete dechlorination. Because DCEs and VC are themselves carcinogenic and mutagenic reagents (Maltoni & Gurengerich, 1974), the anaerobic dechlorination of TCE is not desirable in the environment.

2.3.5 Aerobic co-metabolism of TCE

Aerobic degradation of TCE has been the subject of study since 1985, when it was found for the first time that TCE can be degraded to carbon dioxide under aerobic conditions in soil columns supplied with natural gas (77% methane) (Wilson and Wilson, 1985), without the formation of toxic products. Since then, more work has been dedicated to the study of TCE co-metabolism under aerobic conditions.

As mentioned above, the degradation of short chain chlorinated aliphatic hydrocarbons is very much dependent on their structures, especially the content of chloride ion in the molecule. Under aerobic conditions, less chlorinated compounds, e.g. chloroform, vinyl chloride, trans- and cis-dichloroethylene can serve as the sole carbon and energy sources for certain bacteria (Wijngaard et al., 1993; Bradley & Chapelle, 2000; Vercé et al., 2000). Compounds with higher degree of chlorination like TCE and TCA can only be transformed co-metabolically. Carbon tetrachloride

(CT) and tetrachloroethylene (PCE), except in a single case (Ryoo et al., 2000), are generally considered as non-biodegradable under aerobic conditions.

Therefore TCE is of special importance, as it is the direct product of anaerobic PCE degradation, and the precursor of DCE isomers and VC. It also shares similar degradation mechanism with chloroform and DCEs. In addition, TCE itself is produced in large quantity, applied widely and of potential hazard. As a result, TCE has become the model compound to study aerobic co-metabolism, especially that of short chain chlorinated aliphatic hydrocarbons.

2.3.5.1 Primary substrates supporting TCE co-metabolism

Numerous microorganisms and their relative enzyme systems have been documented to have the ability to co-oxidize TCE. The primary substrates supporting these microbes are shown below in Figure 2.4.

Fig. 2.4. Primary substrates supporting TCE co-oxidation.

Among the large variety of organic and inorganic compounds in nature, only a few can be used as primary substances to induce the necessary enzymes for TCE oxidation catalysis in certain microorganisms. Correspondingly, not all the enzymes can perform the co-oxidation of TCE. The co-oxidation of TCE is catalyzed by a few monooxygenases and dioxygenases of broad specificity, which are originally designed for the oxidation of the primary substrates, e.g. methane, phenol or ammonia. These enzymes usually do not specifically cleave carbon-chlorine bonds, but produce unstable intermediates that decompose and release chloride by chemical decomposition (Janssen & Witholt, 1992). These enzymes can be classified as alkane monooxygenases, alkene monooxygenases, aromatic ring mono- and di-oxygenases, and ammonia monooxygenases.

Chlorinated methanes, ethanes and ethenes can all be co-oxidized by these enzymes, but it has been reported that aromatic compounds are more suitable in treating chlorinated ethylenes, while microorganisms grown on saturated hydrocarbons (methane and propane) are better candidates to treat chlorinated methanes and ethanes (Semprini, 1997). This is presumably due to the similarities between the structures of the primary substrates and the co-substrates. Methane and propane are very close in structure to dichloromethane, chloroform and chloropropanes. Chlorinated ethylenes and aromatic compounds share the similarity of π bonds between two carbon atoms. Thus enzymes catalyzing the degradation of the primary substrates can also recognize the co-substrates, which results in fortuitous degradation of the latter.

2.3.5.2 Enzyme reactions and representative strains of the oxygenases

The non-specific enzymes are originally designed for the catalysis of certain steps in the primary substrate degradations. In order to better understand the degradation of TCE, it is important to know which enzyme catalyzes the reaction and how the reaction proceeds. There may be a large diversity in the microorganisms capable of oxidizing certain growth substrate, but only a few strains have the ability to oxidize TCE at a considerable speed (Nelson et al., 1988; Oldenhuis et al., 1989). These strains are mostly isolated from TCE contaminated sites and are then used as model

microorganisms for TCE co-metabolism tests. The representative strains for TCE co-metabolism and catalysis reactions of their physiological substrates are shown in the following table (Table 2.1).

Table 2.1. Representative strains for TCE co-metabolism and catalysis reactions of their physiological substrates

Enzyme	Physio-logical substrates	Catalytic reactions of physiological substrates	Representative strains	Reference
Ammonia monooxygenase (AMO)	ammonia	$\text{NH}_3 + \text{O}_2 + 2 \text{H}^+ \longrightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O}$	<i>Nitrosomonas europaea</i>	Keener & Arp, 1993
Methane monooxygenase (MMO)	methane	$\text{CH}_4 + \text{O}_2 + 2\text{H}^+ \longrightarrow \text{CH}_3\text{OH} + \text{H}_2\text{O}$	<i>Methylosinus trichosporium</i> OB3b; <i>Methylococcus capsulatus</i> (Bath); <i>Methylobacterium</i> 68-1	Alvarez-Cohen & McCarty, 1991c
Alkane monooxygenase	propane n-butane	$\text{CH}_3\text{CH}_2\text{CH}_3 + \text{O}_2 + 2\text{H}^+ \longrightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{OH} + \text{H}_2\text{O}$ $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_3 + \text{O}_2 + 2\text{H}^+ \longrightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{OH} + \text{H}_2\text{O}$	<i>Rhodococcus</i> sp.; <i>Pseudomonas butanovora</i> , <i>Mycobacterium vaccae</i> JOB5; butane grown strain CF8	Malachowsky et al., 1994; Hamamura et al., 1997

Table 2.1: Representative strains for TCE co-metabolism and catalysis reactions of their physiological substrates (continued)

Alkene monooxygenase	ethylene propylene butylenes	$\text{CH}_3\text{CH}=\text{CH}_2 + \text{O}_2 + 2\text{H}^+ \longrightarrow$ $\text{CH}_3\text{CH}(\text{O})\text{CH}_2 + \text{H}_2\text{O}$	<i>Xanthobacter</i> strain Py2	Ensign et al., 1992
Isoprene monooxygenase	isoprene	not known	<i>Alcaligenes denitrificans</i> subsp. <i>xyloxydans</i> JE75; <i>Rhodococcus</i> <i>erythropolis</i> JE77	Ewers et al., 1990
Toluene 2,3-dioxygenase (TDO)	Toluene, phenol, <i>o</i> - <i>m</i> -cresol		<i>Pseudomonas putida</i> F1; <i>Pseudomonas putida</i> UV4	Heald & Jenkins, 1994; Leahy et al., 1996
Toluene 2-monooxygenase (T2MO)	as above		<i>Burkholderia cepacia</i> G4	Newman & Wackett, 1995

Table 2.1: Representative strains for TCE co-metabolism and catalysis reactions of their physiological substrates (continued)

Toluene 3-monooxygenase (T3MO)	as above		<i>Ralstonia pickettii</i> PKO1	Olsen et al., 1994
Toluene 4-monooxygenase (T4MO)	as above		<i>Pseudomonas mendocina</i> KR1	Whited & Gibson, 1991
Phenol hydroxylase (PH)	as above		<i>Ralstonia eutrophus</i> JMP 134; <i>Comamonas testosteroni</i> ; <i>Pseudomonas putida</i>	Harker & Kim, 1990; Futamura et al., 2001a

Table 2.1: Representative strains for TCE co-metabolism and catalysis reactions of their physiological substrates (continued)

Isopropylbenzene dioxygenase	isopropylbenzene (cumene)		<i>Rhodococcus erythropolis</i> BD2	Dabrock et al., 1992
Some unknown enzyme along the 2,4-D degradation pathway	2,4-dichlorophenoxyacetic acid	not known	<i>Ralstonia eutrophus</i> JMP134	Harker & Kim, 1990

Methane monooxygenases (MMOs) catalyze methane to methanol. The subsequent degradation of methanol to formaldehyde, formate and carbon dioxide provides methanotrophs with carbon and energy. There are two kinds of MMOs: soluble and particulate methane monooxygenase (sMMO & pMMO). All methanotrophs are found to produce pMMO (DiSpirito et al., 1992), which is membrane bound, but only some strains are able to express sMMO under copper limitation ($<0.25 \mu\text{mol g biomass}^{-1}$) (Tsien et al., 1989). MMO catalyzes a wide range of oxidative reactions including the hydroxylation of alkanes, epoxidation of alkenes and oxidation of ethers, halogenated methanes, and cyclic and aromatic compounds (Colby et al., 1977; Patel et al., 1982). It has been found that sMMO has a wider substrate specificity than that of pMMO, and the degradation rates of halogenated hydrocarbons are much higher for sMMO in cell suspensions (van Hylckama Vlieg 1996 & 1997).

Ammonia monooxygenase oxidizes ammonia to hydroxylamine, which is further oxidized to nitrite by hydroxylamine oxidoreductase. The ammonia monooxygenase in *Nitrosomonas europaea* was found also to catalyze the oxidation of carbon monoxide (CO), methane, cis- and trans-2-butene, methanol, ethylene, propylene bromoethane and trichloroethylene (Vannelli et al., 1990). Nitrifying bacteria and methanotrophs share similarity in their substrate specificities and their inability to grow on substrates other than ammonia and methane, respectively. As they are both autotrophs, their growth rates are generally low.

Several strains show the ability to cooxidize a number of CAHs when grown on propane, butane, C₂~C₉ alkanes and C₂~C₄ alcohols but not alkenes and sugars. These enzymes are classified as alkane monooxygenase (Malachowsky et al., 1994; Hamamura et al., 1997).

While alkane monooxygenases catalyze their physiological substrates by hydroxylation, the catalysis by alkene monooxygenases clearly proceeds through a different pathway. Bacteria grown with simple olefins (ethylene, propylene and butylene) as their sole carbon and energy sources initiate the oxidation of alkenes via epoxides, the latter

undergoing further enzymatic oxidations to yield energy and carbon. Another evidence of the different pathways of MMO, AMO and alkene monooxygenase is that the former two can catalyze the oxidation of alkenes, but the latter one does not oxidize saturated hydrocarbons (Ensign et al., 1992).

There are four kinds of toluene oxygenases that can be involved in TCE co-metabolism: one dioxygenase and three monooxygenases. Toluene-2-monooxygenase (T2MO) oxidizes toluene on the ortho- and meta- position stepwisely, forming the product 3-methylcatechol. Toluene-3-monooxygenase (T3MO) first oxidizes toluene on the meta-position, followed by the oxidation on the ortho-position. Toluene-2,3-dioxygenase (TDO) incorporates a molecule of oxygen on the meta-and ortho-position simultaneously. The product of these two enzymes is also 3-methylcatechol. Toluene-4-monooxygenase (T4MO) first forms *p*-cresol, the methyl group of the latter is then oxidized, producing *p*-hydroxybenzoic acid, which is further catalyzed to protocatechuate. These substances subsequently undergo ring fissions catalyzed by catechol-1,2-dioxygenase (ortho-pathway) or catechol-2,3-dioxygenase (meta-pathway) to yield energy (Shields et al., 1991).

There is no strict discrimination between the toluene monooxygenases and phenol hydroxylase. All the enzymes catalyze the oxidation of substituted aromatic rings. Phenol oxidizers can grow on toluene, and toluene dioxygenase was also found to function as a monooxygenase which can catalyze phenol (Spain et al., 1989). Phenol hydroxylase oxidizes phenol on the ortho-position, forming catechol. The latter then undergoes ring cleavage via ortho- or meta-pathways.

2.3.5.3 Exogenous electron donors for TCE co-metabolism

Oldenhuis et al. (1991) suggested that one NADH is needed in the first step of TCE co-metabolism, that is, the oxidation of TCE to TCE epoxide. But this consumption will not be replenished, for the subsequent degradation of TCE is mainly by chemical means

(Dolfing, 1993). Therefore if the microorganisms are in a resting state, the cell can be provided with other substances to regenerate the NADH consumed in TCE degradation. Formate, as a product along the methane degradation pathway, is often used for this purpose with methanotrophs (Alvarez-Cohen & McCarty, 1991b). For a similar reason, butyrate is used for butane oxidizers (Hamamura et al., 1997). With nitrifying bacteria, hydrazine (N_2H_4) is an alternate substrate for hydroxylamine oxidoreductase (HAO) and an effective electron donor, with N_2 as the product (Keener & Arp, 1993). Glutamate is used for toluene oxidizers (Winter et al., 1989), and formate, lactate and acetate for phenol oxidizers (Hopkins et al., 1993a). Lactate is believed to be a good choice, because it has the least repressive effect on the induction of toluene degradation (Park et al., 2002). But it was also found that while lactate might enhance cell growth, the TCE transformation rate was decreased (Leahy et al., 1996).

2.3.5.4 Pathway of TCE aerobic co-metabolism

The detailed mechanism of the steps in TCE degradation pathway is not yet very clear, but it is generally concluded that monooxygenases and dioxygenases oxidize TCE through different pathways.

TCE is catalyzed by a monooxygenase to form an unknown intermediate (Figure 2.5). The intermediate is diverted between TCE epoxide (through epoxidation) and an aldehyde. The latter undergoes chlorine ion migration, with the stable end-product of chloral. TCE epoxide may be subsequently metabolized by microorganisms or degraded chemically. Studies tracing the products of TCE degradation have revealed that the main products are carbon monoxide, formate, glyoxylate and dichloroacetate (Fox et al., 1990; Li & Wackett, 1992; Newman & Wackett, 1997), which are consistent with the products of the chemical reaction of TCE epoxide in aqueous solution. Indirect evidence is also obtained by online monitoring of TCE epoxide fate, which showed that TCE epoxide degradation was not biologically mediated (van Hylckama Vlieg et al., 1996). Thus, it is generally concluded that the degradation of TCE epoxide does not involve enzymatic reaction.

Fig. 2.5. Pathway of TCE degradation by monooxygenase (Fox et al., 1990).

As shown in Figure 2.6, the reaction pathway catalyzed by a dioxygenase clearly doesn't involve epoxidation (Li & Wackett, 1992). The mechanism of TCE degradation by dioxygenases is not well understood. Besides, TCE degradation pathways and products not only vary with different enzymes, but also are related to intra- and inter-cellular environments.

Fig. 2.6. Pathway of TCE degradation by dioxygenase.

2.3.5.5 Complete mineralization of TCE

According to Dolfig (1993), complete mineralization of TCE proceeds via the following stoichiometry:

Complete mineralization is the ideal result of TCE degradation. As stated above, incomplete dechlorination may occur, with the stable products of acyl chlorides and carbon monoxide, etc. Yet complete mineralization has been observed with mixed cultures enriched on methane, propane, toluene or phenol (Chang & Alvarez-Cohen, 1995b); pure cultures of *Xanthobacter* sp. strain Py2 (Reij et al., 1995), *Nitrosomonas europaea* (Hyman et al., 1995), *Pseudomonas cepacia* G4 and G4 PR1, *Pseudomonas mendocina* KR1, *Pseudomonas putida* F1, and *Methylosinus trichosporium* OB3b (Sun & Wood, 1996), where the products formed were carbon dioxide and chloride ions.

2.3.5.6 Toxic effect of TCE co-metabolism

In addition to the common features characteristic of all co-metabolism reactions, TCE and other CAH degradations are accompanied by a unique phenomenon, the turn-over dependent toxicity on microorganisms.

The phenomenon was first observed in a resting cell system, where the quantity of TCE degraded by microorganisms was limited (Henrysson and McCarty, 1993). The disappearance of TCE might proceed for a period of time, but the transformation rate gradually approached zero.

The cause of such phenomenon was first postulated to be the depletion of reducing energy, NADH. However, in studies with methanotrophs, where formate can be used as a non-competitive, non-growth-supporting electron source, it was found that the addition of formate in resting cells did enhance TCE degradation, but the degradation was still not sustainable (Alvarez-Cohen & McCarty, 1991c). This finding suggested that the reducing energy limitation did play some role in the finite TCE transformation in resting cells, but other factors existed. This factor is referred to as the toxicity of TCE degradation.

Further studies were conducted to explore the effect of TCE transformation toxicity. Various pure cultures with the ability of TCE degradation were exposed to TCE in resting states, after which the cells were washed and resuspended in fresh medium. The TCE transformation rates of these cultures during TCE exposure were found to gradually approach zero. After TCE transformation, the washed cells showed decreased cellular activities, e.g. substrate degradation rate, TCE degradation rate and endogenous respiration activity. The toxic effect was not specific towards the transforming enzymes. Instead, the specific TCE degradation ability (Oldenhuis et al. 1991), the specific primary degradation ability (Chu & Alvarez-Cohen, 1996), primary substrate-dependent oxygen demand (Hyman et al., 1995), specific enzyme activity, general respiratory activity (Chu & Alvarez-Cohen, 1999) and cell viability (Heald & Jenkins, 1994) were all compromised. The worst situation is that cells were totally inactivated after transforming a certain amount of TCE, exhibited as the diminished TCE and primary substrate

degradation rate, and the nearly zero growth and respiration rate. The totally inactivated cells can be considered as dead, as further incubation with their growth substrate could not restore their activities (Chu and Alvarez-Cohen, 1999). The amount of TCE that could be degraded per unit of biomass before total inactivation occurs was defined as the TCE transformation capacity (T_c).

Two things must be pointed out in measuring T_c . Firstly, cells should be in a complete resting state, i.e. no primary or growth-supporting substances is added to cells. Secondly, reducing energy limitation should not occur during the degradation of TCE. The cells should be provided with adequate reducing energy sources, whether in the form of direct addition of NADH, or by supplying an exogenous electron donor to obtain the unbiased value of transformation capacity.

2.3.5.7 The nature of the toxicants from TCE degradation

Different researchers have reached the same conclusion that it is not TCE itself, but some product(s) of TCE degradation that are the agents causing the toxic effect (Alvarez-Cohen & McCarty, 1991b; Mu & Scow, 1994; van Hylckama Vlieg et al., 1997). This inactivation effect is referred to as transformation product toxicity, in short, TPT (Dolan & McCarty, 1995). The mechanism of the toxicity is believed to be the covalent binding of the toxicant to the cellular materials in the TCE degrading cells. While bond by the toxicants, the structures of the cell components could be modified, and the functions lost.

The nature of such agents and the mechanism of the toxicity are not well understood, but it is postulated that neither TCE nor TCE epoxide or chloral is responsible for the toxic effect, and the modifying reagent(s) is a diffusible intermediate derived from the non-enzyme-catalyzed hydrolysis or isomerization of TCE epoxide, such as glyoxyl chloride, formyl chloride, dichloroacetyl chloride or dichlorocarbene (Fox et al., 1990). The toxicants are highly electrophilic compounds which can affect TCE degrading microorganisms either by non-specific covalent modification of the specific enzymes and

other cellular materials, or by alkylating nucleophilic groups in nucleic acids and proteins, thus inhibiting essential metabolic processes (Rasche et al., 1991).

Studies revealed that the toxicants are short-lived and diffusible. After cells had transformed TCE, the supernatant was purged and incubated with fresh cells. No negative effect was observed (Chang & Alvarez-Cohen, 1995b; Yeager et al., 2001b) for the fresh cells, suggesting no residue toxicity, presumably because the toxicants had already decayed in the process of wash, centrifuge and resuspension. However, when TCE-degrading and non TCE-degrading cells (e.g. yeast) are incubated together with TCE, the latter seemed to be impaired by TCE transformation as well, although they didn't degrade TCE (Reij et al., 1995; Yeager et al., 2001b). A decrease of cell culturability of *Burkholderia cepacia* G4, and the fermenting activity of *Saccharomyces cerevisiae* was observed, suggesting that the toxicants did leave the enzyme active sites and diffused out of cell membrane to the bulk liquid.

Because the toxicants are diffusible and non-specific, the toxic effect on microorganisms is dependent not only on the degradation pathways and products, but also on the intra- and inter-cellular environment. If cells are protected by the diffusion limitation, or the toxicants from TCE degradation experience detoxification before binding to cellular materials, the cells can be less affected by the toxic effect. For instance, purified enzymes sMMO, TDO and T2MO were covalently modified when incubated with TCE, but only whole cells of *Methylosinus trichosporium* OB3b (sMMO) and *Pseudomonas putida* F1 (TDO) exhibited toxicity in vivo (Oldenhuis et al., 1991; Wackett & Householder, 1989). In contrast, *Burkholderia cepacia* G4 (T2MO) was not significantly affected by the TCE transformation product toxicity (Folsom et al., 1990; Shields & Reagin, 1992).

2.3.5.8 Long term effect of TCE degradation toxicity

Because TCE transformation can harm cells that carry out this process, long term exposure to TCE can cause negative changes in microbial communities

When a pure culture of *Burkholderia cepacia* G4 was incubated with toluene alone or toluene plus TCE, it was observed that the specific maintenance energy requirement was increased to 4-fold when TCE was present, i.e. with the same amount of toluene, TCE degrading bacteria produced 1/4 amount of biomass. When toluene supply was cut off from the culture but TCE still added, mutants appeared which could degrade neither toluene nor TCE, i.e. the TCE-degrading enzymes were no longer expressed. These mutants were believed to be feeding on dead cell materials. It was postulated that TCE transformation product toxicity exerted a selective pressure against TCE degrading cells, causing the loss of the plasmid involved in T2MO synthesis (Mars et al., 1996).

The competitive behavior of a mixture of several toluene degrading strains in response to TCE exposure was further studied (Mars et al., 1998). There were four toluene degrading strains in the mixture, two of which degraded TCE, and the other two didn't. After exposure to TCE and low concentration of toluene for several weeks, strains not able to degrade TCE became dominant. Because TCE degradation damages cellular materials, cells that don't transform TCE will have a selective advantage over those who do in a microbial community. The phenomenon was also attributed to the competitive inhibition of TCE on toluene degradation in addition to the toxic effect.

These findings suggested a suitable pattern of primary substrate feeding in TCE co-metabolism. As a low primary substrate supply causes serious competition among different strains in a mixed culture, the primary substrate should be present in abundance for the TCE degraders to survive. In a continuously fed reactor, the concentration of the substrate is generally low, but a reactor operated in batch mode will generate a much higher initial concentration. Therefore it is possible that the batch mode is more favorable for TCE co-metabolism.

2.3.5.9 Recovery of cells from TCE turn-over dependent inactivation

The inactivation of cells by TCE TPT was suggested to be irrevocable by some researchers (Alvarez-Cohen & McCarty, 1991c), that the toxicants once bond to the

cellular materials would not detach and the modified cellular components totally lost their functions. However, in certain circumstances, incubation of cells that had transformed TCE with their primary substrates sometimes could result in the partial recovery of the cellular activities.

Two thresholds related to the recovery ability have been found for the quantity of TCE transformed by certain amount of cells.

The first was postulated by Chu and Alvarez-Cohen (1999), which was equal to the T_c of certain microbes. When cells of *Methylosinus trichosporium* OB3b had spent their TCE transformation capacity, the respiratory activity of the cells reduced to <5% of the original value, and even 7 days incubation with methane didn't recover the cells from damage. This threshold is referred to as "recovery threshold", below which cell recovery is possible.

The second threshold was found with *Burkholderia cepacia* G4 (Yeager et al., 2001b). The cells' culturability was determined after TCE degradation, and was found not to be significantly affected when the TCE transformed to biomass ratio was less than $0.5 \mu\text{mol mg cell}^{-1}$ (equal to 66% T_c). But above that value, toluene degrading ability and general cellular activity were compromised. Thus it is referred to as "activity threshold", above which the toxic effect will impair the cells to a significant degree.

The two thresholds affecting the recovery of TCE damaged cells may not simultaneously exist for certain microbes, because the two studies mentioned above were conducted with different strains and experimental conditions. But if they do exist, there would be a potential of utilizing as much as the degradation potential in cells, without causing serious inactivation.

2.3.5.10 The mechanism of recovery

Two mechanisms are possible for the cellular recovery from TCE TPT, by producing new cells or by synthesizing new enzymes and cellular materials. Studies with *Nitrosomonas europaea* have revealed that cells recovered by resynthesis of new proteins within preexisting cells rather than by an uncharacteristically rapid division of a subset of noninactivated cells (Hyman et al., 1995). The recovery of cells from TCE TPT involved de novo synthesis of proteins, while DNA repair in *Burkholderia cepacia* G4 required energy upon degradation of TCE (Yeager et al., 2001a). In conclusion, excessive energy is needed to repair the modified cellular materials, whether it is proteins or DNA molecules, therefore a substance providing energy should be present during recovery.

Hence the traditional model of TCE TPT and recovery can be expressed as: cell growth on the primary substrate—TCE transformation—inactivation and partial loss of cellular activities—incubation with the primary substrate again—degradation of the primary substrate and obtaining energy—synthesis of new cellular materials to replace the inactivated ones—the next round of TCE degradation. The severity of inactivation is indicated by the amount of TCE transformed by microbes, and when cells have transformed TCE equal to their transformation capacity, they are considered as dead. The inactivation is in positive relationship with the amount of TCE transformed, either linearly or exponentially (Chu & Alvarez-Cohen, 1999; Yeager et al. 2001b).

However, some researchers found such a model over simplified and developed more sophisticated models to describe the enzyme inhibition, inactivation and recovery in TCE co-metabolism (Ely et al, 1995). Instead of relating TCE TPT to cell death, the toxic effect was studied at the enzyme level. It was proposed that even during TCE turnover, recovery of enzyme from TCE TPT is possible, and the higher degree of inactivation there is, the larger is the driving force for the microbes to obtain energy for recovery.

Such a model, however, is still not sufficient to explain all the TCE degradation phenomena. An important reason is that the TCE transformation products don't inactivate only the enzyme that catalyzes the reaction. Rather they attack the cellular materials

indiscriminately. In a study with *Burkholderia cepacia* G4, it was found that the reduction of general respiratory activity outpaced that of the toluene-degradation activity, indicating the TCE TPT caused more severe damage to other cellular materials than to the toluene 2-monooxygenase that transformed TCE (Yeager et al., 2001a). Therefore models only considering the inactivation of the non-specific enzyme are not conclusive. Monoro et al. (2004) reported the immediate restoration of toluene-degradation activity after TCE transformation, and proposed a pseudo-inactivation theory. That is, the toxic products don't diffuse out of the enzyme after they are generated, and their binding to the enzyme is reversible. When the primary substrate is added after TCE degradation, the toxicants are released from the enzyme, and the degradation of the primary substrate immediately resumes.

As the TCE transformation product toxicity is a profound phenomenon, the existent models may only be able to describe certain aspect of it, and the whole picture is lacking.

2.3.5.11 Parameters in evaluating TCE degradation

Four parameters are important in evaluating the TCE transformation ability of microorganisms:

- ❖ T_r : transformation rate
- ❖ T_e : transformation extent
- ❖ T_c : transformation capacity
- ❖ T_y : transformation yield

Transformation rate (T_r) is the speed with which cells transform TCE. When TCE degradation is described by Michaelis-Menten kinetics, the rate can be expressed as the pseudo-first order rate: $k_1 = V_{\max}/K_s$, provided that TCE concentration is low. The unit of T_r is usually mg TCE transformed mg SS⁻¹ h⁻¹.

Transformation extent (T_e) stands for the degree to which TCE can be transformed. In the case of complete mineralization, it can be expressed as the degree of dechlorination. To measure T_e , the quantity of chlorides released from TCE is a good indicator. T_e can be expressed in the unit of percentage (%) of the original TCE amount that is transformed or mineralized.

T_c , or transformation capacity, was defined earlier. The unit for T_c is mg TCE transformed mg SS^{-1} .

T_y is only useful in growing or semi-growing cells. The meaning of transformation yield is the amount of TCE that can be degraded by cells growing on certain amount of growth substrates, so the unit is mg TCE degraded $\text{mg growth substrate}^{-1}$. When T_c and y (growth yield) of certain cells are known, T_y can be calculated.

Generally a higher T_c and T_y are advantageous for TCE transforming microorganisms. A high T_c indicates that less cells will be inactivated by the degradation of TCE. A high T_y is related to less growth substrate needed to recover or grow the cells, thus the lower cost when the growth substrate is a natural resource (e.g. methane). As to T_e , as high as possible a T_e is favorable when complete dechlorination occurs; but when transformation of TCE results in the stable accumulation of toxic substances in microorganisms, a lower transformation extent is also acceptable. T_r is also a controversial parameter. Faster transformation is certainly desired, but sometimes a high degradation rate is also accompanied by faster inactivation of cells.

2.3.5.12 Other effects of TCE on the co-metabolism systems

❖ Solvent stress

If TCE exerts only the transformation products toxicity on cells, the effect of initial concentration of TCE on its degradation should be adequately described by Michaelis-Menten kinetics. But it was found that when TCE concentration in the liquid phase exceeded a certain threshold, degradation of TCE would not occur at all, and deviation from Michaelis-Menten kinetics was observed. Oldenhuis et al. (1991) defined it as the

acute toxicity threshold, in order to differentiate it from the TPT. The dependence of transformation capacity, which should be theoretically only relevant to the transformed amount of TCE, on initial TCE concentrations was also observed (Alvarez-Cohen & McCarty, 1991c; Hyman et al., 1995). These findings suggested that effect of initial TCE concentration should play some role in TCE degradation.

Park et al. (2002) further related this effect to solvent stress. Organic solvents with a log K_{OW} between 1.5 and 3 may generally cause cytoplasmic membrane-associated toxicity. The log K_{OW} of TCE is 2.61, therefore it may cause a concentration-dependent stress on the cells when presented in liquid phase. This hypothesis was verified by using PCE (tetrachloroethylene), a compound not aerobically degradable but with a similar chemical structure and log K_{OW} to TCE, as the solvent. Reduced viability of cells was observed with cells exposed to PCE, indicating the presence of solvent stress.

❖ Induction of TCE and primary substrates degrading enzymes

TCE had always been viewed as a simple co-substrate that is only oxidized fortuitously by the enzyme, and therefore should have no effect on the expression of the non-specific enzymes. However, Heald and Jenkins (1994) found for the first time that TCE could induce the degradation of itself, as well as that of toluene. This finding was further supported by McClay et al. (1995), Leahy et al. (1996), Shingleton et al. (1998) and Park et al. (2002). The phenomena observed were that when cells were pre-exposed to TCE plus some non-inductive substrates (e.g. lactate), cells exhibited toluene degradation activity at around 40%~80% of the level shown by the cells induced by toluene. This suggests that TCE can induce its own degradation and the expression of the non-specific enzyme, although not so efficiently as toluene.

❖ Induction of tolerance to TCE toxicity

Park et al. (2002) also found that when both the TCE induced and uninduced cells had degraded the same amount of TCE, the reduction in growth rate for TCE-induced cells was smaller than that for uninduced cells. This could be attributed to the effect of TCE pre-exposure in leading to an increased tolerance to TCE-mediated stress. The possible

explanation is that TCE pre-exposure selectively enriched those cells that can survive TCE solvent stress, or that solvent stress responses are also linked to the regulation of T3MO pathway expression.

❖ Other effects

As a combination of the stated effects above and some other unknown factors, TCE may exert a profound selection pressure on the microbial community that is exposed to TCE for a long term. Ayala-del-Río et al. (2002) conducted a long term experiment, in which two reactors were operated in identical sequencing batch feeding patterns, but one was exposed to phenol alone and the other phenol plus TCE. Contrary to the findings of Mars et al. (1996 & 1998), TCE degradation ability was not diminished in the phenol plus TCE reactor in a period of 900 days. The subcultures from the phenol plus TCE reactor gave lower but more stable performance in phenol and TCE degradation, corresponding to a distinction between the microbial communities in the two reactors. This suggests a complicated interaction between TCE solvent stress, TPT stress, induction and repression of phenol hydroxylase and microbial community structure.

2.4 Phenol

Phenol is an aromatic compound and a widely used industry chemical, which is commonly found in wastewaters from oil refineries, petrochemical plants, coking plants and phenol resin industry (Watanabe et al., 1996a). Like most aromatic compounds, phenol is a toxic substance, with a log K_{OW} value of 1.46. It affects the metabolic activities of cells by decreasing the integrity and function of cell membrane (Davidson and Branen, 1981). The degradation of phenol in natural environment is common because the structure of benzene ring is not xenobiotic. Many soil bacteria have the ability to degrade phenolic compounds, but due to its toxicity, high phenol concentration can still exert inhibitory effect on unacclimated cells (Dagley, 1985).

2.4.1 The degradation pathway of phenol

As shown in Figure 2.7, phenol degradation is initiated by phenol hydroxylase (PH), followed by ring cleavage on the meta or ortho position by catechol-2,3-dioxygenase (C23O) or catechol-1,2-dioxygenase (C12O), respectively. The rate limiting step is believed to be the hydroxylation reaction (Shingler et al., 1989).

Fig. 2.7. Degradation pathway of phenol (Dagley, 1985).

2.4.2 The kinetics of phenol degradation

As an inhibitory substrate on its own degradation, phenol transformation kinetics can not be adequately modeled by simple Michaelis-Menten kinetics. Instead, Haldane or Andrews kinetics, which incorporates substrate inhibition effect is usually used (Bailey & Ollis, 1986):

$$V = \frac{V_{\max} \times S}{K_s + S + \frac{S^2}{K_i}}$$

where V is the specific degradation rate, $V_{\max}$, S and K_S are the maximal specific degradation rate, concentration of the substrate and the half-saturation constant, respectively; K_i is the inhibition constant of the substrate.

Other models have also been developed to describe the degradation of inhibitory substrates (Aiba et al., 1968; Edwards, 1970; Han & Levenspiel, 1988; Sokol & Howell, 1981; Wayman & Tseng 1976; Webb, 1963; Yang & Humphrey, 1975; Yano, 1966). However, as the models gave similar predictions (Onysko et al., 2000), the one with the least parameters (Haldane's equation) can be adopted for its simplicity.

2.4.3 Phenol as a primary substrate in TCE co-metabolism

Previous studies have examined the use of phenol as the primary substrate for TCE co-metabolism. Laboratory studies (Folsom et al., 1990; Harker & Kim, 1990; Fan & Scow, 1993; Krumme et al., 1993; Chang & Alvarez-Cohen, 1995b; Fujita et al., 1995; Sun & Wood, 1996; Shih et al., 1996; Futamata et al., 2001a, 2001b) and field trials (Hopkins et al., 1993b; Hopkins & McCarty, 1995) have come up with results summarized as follows.

2.4.3.1 Effect of phenol degradation pathway on TCE oxidation

Only one enzyme along the phenol degradation pathway is involved in TCE co-metabolism: the phenol hydroxylase (PH), which catalyzes the first step of phenol oxidation. Therefore theoretically, TCE degradation kinetics and extents should be closely associated with the activity of this enzyme. However, in the work of Fujita et al. (1995), who used genetically engineered strains carrying cloned phenol catabolic genes from *Pseudomonas putida* BH, phenomena defying this theory were observed.

First, wild type *P. putida* BH could degrade TCE when grown with phenol, *o*-cresol, *m*-cresol or *p*-cresol, which induced C23O (*meta*-cleavage); but hardly exhibited any activity in TCE degradation when grown with aromatic compounds (benzoate, protocatechuate etc.) that induced C12O activity or induced neither C12O nor C23O.

Second, when various fragments containing phenol/cresol catabolic genes were introduced into *P. putida* KT2440, the host strain could degrade TCE to an extent when expressing only phenol hydroxylase (PH). When it expressed the 3 additional meta-fission enzymes: C23O, 2-hydroxymuconic semialdehyde hydroxylase (2HMSH) and 2-hydroxymuconic semialdehyde dehydrogenase (2HMSD), the transformation extent was much higher. Therefore, it can be inferred that activity of enzymes other than PH along the phenol degradation pathways may play some role in TCE co-metabolism.

One possible explanation for this phenomenon was that the toxic effect in *Pseudomonas* might be reduced by the coexistence of meta-fission enzymes; or rather that as the degradation was conducted with whole strains, the presence of ring-cleavage enzymes could provide the cells with more energy and reducing equivalents for them to recover from toxicity and depletion of reducing energy.

2.4.3.2 Effect of phenol degradation kinetics on TCE co-metabolism

Watanabe et al. (1996b) observed that when modeled by Haldane's formula, bacteria could be classified into three groups according to their kinetics of phenol degradation. Group I bacteria exhibited low apparent K_S ($<1 \mu\text{M}$) and K_i ($<300 \mu\text{M}$); group II: K_i around $1400 \mu\text{M}$; group III: K_i around $3000 \mu\text{M}$ and the apparent K_S higher than that of group II. This finding was further tested by Futamata et al. (2001a), where those strains were used to degrade TCE. A positive correlation between K_S for phenol and K_S for TCE was found, i.e. bacteria exhibiting high K_S for phenol showed high K_S for TCE, and vice versa. The strains were again classified into three groups, and the low K_S group bacteria were found to have a high TCE degradation activity at low TCE concentration (Futamata et al., 2001b). Thus the kinetics of phenol and TCE degradation were directly related, and the low K_S bacteria were suggested to be superior in TCE bioremediation application.

2.4.3.3 Effect of phenol feeding pattern on TCE co-metabolism

Futamata et al. (2001b) suggested, based on their findings on kinetic groups, that low K_S group bacteria were more suitable in TCE degradation, because they could degrade TCE

to a high extent and with a high rate. Those strains were all isolated from a continuous enrichment culture, while the moderate and high K_s groups were from batch cultures. Therefore it seems that a continuous culture is favorable to cultivate TCE degrading cells.

However, this suggestion was solely based on the kinetic behavior, therefore may not be conclusive. Shih et al. (1996) compared the effects of 4 distinct phenol feeding patterns on TCE transformation. Reactors operated in continuous or semi-continuous pattern produced cells much less capable of TCE degradation, and biomass taken from the pulse-fed reactor gave the best performance. Similar phenomena were observed by Futamata et al. (2003), that periodical feeding of phenol resulted in a complete degradation of TCE, so the suggestion was made that intermittent supplement of low concentrations of phenol increases the population of low- K_s group bacteria.

2.4.3.4 Effect of phenol concentration on TCE-degradation

In a resting cell system, where phenol and TCE are not simultaneously present, the competitive inhibition will not be significant, but the concentration of phenol to which cells are adapted to may still have some effect on TCE degradation.

From the findings of Futamata et al. (2001a), it was concluded that the concentration of phenol in a system may selectively enrich some bacteria group with certain kinetic property, therefore affect the TCE degradation kinetics. In real application, different concentrations of TCE may be encountered. To meet different demands of TCE removal, the three kinetics groups of bacteria can be selectively enriched by applying a corresponding phenol concentration.

Keweloh et al. (1990) observed a membrane change in free and immobilized *Escherichia coli* and an increase in respiratory activity when cells were grown in the presence of phenol. This was suggested as an adaptive response to resist phenol toxicity. Jiang et al. (2004a) also observed that, when different phenol loadings were applied to sequencing batch reactors, biomass exhibited changes in structural and metabolic characteristics,

presumably in response to different phenol loadings and concentrations. Therefore it is reasonable to presume that adaptive responses mediated by different phenol concentrations may affect the biomass characteristics, and hence affect its resistance to TCE degradation toxic effect.

In a one-stage reactor where phenol and TCE are intermittently fed, the concentration of phenol added in the recovery phase may be critical for the whole process. In the work of Yeager (2001b), it was discovered that when TCE TPT-harmed cells were incubated with lactate or phenol for recovery, cells in the former case took less time to recover than the latter. It was postulated that damaged cells might be more sensitive to phenol toxicity, and the concentration of phenol which was harmless to natural cells might be fatal to the injured cells.

The observations give rise to two thresholds of the phenol concentration applied in the recovery process. The lower threshold is that the phenol quantity added is just enough for the cells to recover, and is referred to as “recovery threshold”. The higher threshold is the phenol concentration that causes inhibitory or toxic effect to the injured cells, referred to as “toxicity threshold”. After each application of phenol, the cells should be restored to at least the original state of activity (i.e. before inactivation by TCE), or the process will not be sustainable. Meanwhile, the time required for recovery is also very important. The shorter the recovery period is, the higher the overall efficiency a reactor may have. It is possible that the quantity needed to recover cells already results in the toxic effect of phenol. In such a case, multi-dosage of phenol in low concentrations should be applied.

2.4.4 Advantage of phenol as a primary substrate

- ❖ Considerable transformation rate of phenol degraders toward TCE

The degradation rates of TCE by phenol oxidizers are not so high as those of methanotrophs (Chang & Alvarez-Cohen, 1995b; Futamata et al., 2001b), but they degrade TCE much faster than nitrifying bacteria (Vannelli, 1990).

❖ Less stringent demand of phenol degraders on the environment

Methanotrophs are superior in their high TCE degradation rates only when copper is eliminated from the environment, which may not be possible in some cases of bioremediation. But phenol oxidizers do not have such scruples (Chang & Alvarez-Cohen, 1995b)

❖ Less sensitive to toxicity effect

Results obtained with phenol oxidizing mixed or pure cultures generally showed less effect of TCE TPT (Folsom et al., 1990; Hopkins et al., 1993b; Futamata et al., 2001a).

❖ Higher growth rate of phenol oxidizers than methanotrophs

As methanotrophs and nitrifying bacteria are autotrophic microorganisms, their growth rates are quite low, e.g. $< 0.1 \text{ h}^{-1}$ for methanotrophs (Park et al., 1991). As TCE product transformation toxicity harms cells, a higher growth rate may be needed to produce new cells for TCE degradation. Phenol oxidizer with a much higher growth rate ($0.2\sim 0.4 \text{ h}^{-1}$) might be a better candidate.

❖ Advantage over toluene

Toluene is also suitable for TCE co-metabolism but phenol is believed to be superior. First, toluene is more toxic than phenol to microorganisms (Fries et al., 1997); second, toluene is less soluble in water and more difficult to handle, while phenol can be easily prepared and stored in high concentration stock solutions. As toluene is more volatile and toxic than phenol, operation with the latter is easier and safer for the operator.

2.5 Aerobic granules

Aerobic granule is a novel form of microbial aggregate that has attracted intensive research and industry interest in the area of wastewater treatment in the last ten years. It is the granular shaped activated sludge formed in aerobic environment by auto-aggregation of microorganisms, usually in column type reactors and under sequencing batch conditions. The aerobic granules normally found in lab and pilot scale reactors are within the size range of 0.3 to 2 mm, and having various densities, but generally higher than that of common activated sludge flocs.

2.5.1 Formation of granular sludge under aerobic conditions

It is not uncommon in natural or artificial environment that microorganisms attach to each other or other surfaces to form aggregates. Granular sludge cultivated under anaerobic condition in column-type upflow reactors (UASB reactor) has been applied in wastewater and sludge treatment processes for decades. Various aerobic reactors in which microorganisms attach to surfaces of carriers to form biofilm have been constructed for all kinds of applications. Aerobic granules differ from anaerobic granules and aerobic biofilm in that, they are formed under aerobic conditions and do not need carriers for aggregation.

During the cultivation of biogranules, microorganisms attach to each other to form compact, dense, strong and often spherical particles, while retaining their biological activities (Tay et al., 2001a). Anaerobic granules are mostly formed in upflow anaerobic granular sludge blanket reactors (UASB), and anaerobic sequencing batch reactors and biofilm airlift were also found to produce granular sludge (Morgenroth et al., 1997). To date, the most successful processes for aerobic granulation have been conducted in sequencing batch reactors (SBR) (Beun et al., 2000; Liu et al., 2005c). SBR is operated under the mode of aeration and reaction—settling of biomass—decanting of supernatant treated liquid— filling of fresh wastewater—aeration and reaction cycle. Aerobic

granular sludge SBR differ from conventional activated sludge reactors in that the settling time is usually very short, in the scale of minutes instead of hours.

The reason why SBR mode is more efficient in aerobic granule cultivation is not yet fully understood. Various theories have been proposed on the mechanism of aerobic granules formation. They mainly address the phenomenon of aerobic granulation from two aspects. The first focuses on the operational conditions favoring the granulation process, e.g. reactor configuration, operation mode, hydraulic condition within the reactor, organic loading rate, and hydraulic and sludge retention time. The second aspect seeks to find the answer in the intrinsic differences of sludge characteristics between aggregated cells and free cells, which might include the cell surface hydrophobicity/hydrophilicity and extracellular polymeric substances (EPS) secretion. Often these two aspects are interrelated, i.e. change of the operation condition will change the production of EPS.

Beun et al. (1999) proposed a mechanism of fungi-assisting cell attachment for aerobic granulation process, that during the early stage of granulation, free cells would be almost completely washed out due to the short settling time, and only mycelial pellets formed by fungi could remain because of their good settling ability. The pellets grow bigger and more compact with the operation, which could function as a matrix for bacteria to attach. When the fungi pellets are too big, oxygen and substrate limitations occur at the center, causing the breakup. Granules of bacteria colonies that have been attached to the fungi matrix are released and dominate the reactor, as they are then big and compact enough to settle within short time. However, this theory can only apply to reactors seeded with small amount of suspended, poorly settling cells, not to those started up with flocs or small granules as the inoculum. Moreover, the underlying assumption of this theory is that aerobic granules will be formed when flocs are washed out, which itself is doubtful.

Tay et al. (2001a) studied the effect of shear force on aerobic granule formation in bubble column sludge blanket reactors. It was discovered that shear force in the reactors, mainly provided by upflow aeration, had a positive effect on the granule formation process. At the lowest superficial upflow air velocity (0.3 cm s^{-1}), granules were not formed at all.

When the aeration rate was increased above 2.0 L min^{-1} , corresponding to a superficial upflow air velocity of 1.2 cm s^{-1} , stable aerobic granules could be formed and maintained. Therefore it was concluded that shear force played a crucial role in aerobic granulation, and could further influence the structure and metabolism of granules (Tay et al., 2004a).

Hydraulic selection pressure was investigated as a possible controlling parameter in the aerobic granulation operation by Tay et al. (2002c). No nitrifying granules were found in the reactor with a cycle time of 24 h, possibly due to the weak hydraulic selection pressure caused by the long hydraulic retention time. Neither was granulation observed under the cycle time of 3 h, where wash out of sludge occurred, leading to the failure of granulation. The cycle times of 6 and 12 h were discovered to produce healthy and active granules, indicating the importance of hydraulic selection pressure to aerobic granule formation.

The effects of substrates and loadings on aerobic granulation were explored by Moy et al. (2002). Both glucose and acetate were used as benign substrates for granulation under various loading rates. When the organic loading rate (OLR) was increased from 6 to $15 \text{ kg chemical oxygen demand (COD) m}^{-3} \text{ d}^{-1}$, glucose fed granules could sustain, but acetate granules disintegrated above the OLR of $9 \text{ kg m}^{-3} \text{ d}^{-1}$. However, under lower OLR, acetate produced compact and spherical granules, while glucose fed granules exhibited loose and fluffy morphology. Therefore substrate type and loading could influence aerobic granulation to some degree, but might not be the decisive factor.

Wang et al. (2006) studied the effect of reactor volumetric exchange ratio on the granulation process. The volumetric exchange ratio is defined as the ratio of the discharged volume to the total operation volume at the end of one cycle. For example, if a reactor with 2 L of operation volume discharges 1 L of treated liquid after each cycle, the volume exchange ratio will be 0.5. Wang et al. concluded that high volumetric exchange ratios favored rapid aerobic granulation, after studying 4 different ratios from 20% to 80%. The possible reason was that under higher volumetric exchange ratios, more supernatant was discharged every cycle, causing more slow-settling particles to be

washed out. Varying volumetric exchange ratios can be a simple way to control granulation and stabilizing processes, and also can affect the mean size of the biomass in the reactor while coupled with different settling times.

Extracellular polymeric substances (EPS) are materials secreted by cells, which could act as a “glue” to enhance the adhesion between cells and other surfaces. EPS, especially the polysaccharides constituents in EPS have been suggested as the major components in the matrix of biogranules (Schmidt and Ahring, 1994; Liu et al., 2004a). The concentrations of cellular polysaccharides were found to be positively correlated to the stability of aerobic granules (Tay et al., 2001b). Aerobic granulation was concurrent with a sharp increase of cellular polysaccharides normalized to cellular proteins, and this ratio was much higher for granules than for bioflocs. A short cycle time was found to stimulate the production of cell polysaccharides (Tay et al., 2002b), and high upflow air velocity could induce higher secretion of polysaccharides by the granules (Tay et al., 2004a). Volumetric exchange ratios of the aerobic granular sludge blanket (AGSB) reactors also exhibited positive correlation with biomass EPS production (Wang et al., 2006). Therefore the operational conditions were linked to the changes of cellular EPS production.

Cell surface hydrophobicity has been identified as an important factor affecting the approaching and adhesion of cell-to-cell and cell-to-other surfaces (Yaskovich, 1998, Liu et al., 2004b). It was discovered that the hydrophobicity of granular sludge was much higher than that of bioflocs (Tay et al., 2001a). Liu et al. (2003) has shown that the cell surface hydrophobicity increased sharply when granules were being formed. Shear force was found to have a positive effect on cell hydrophobicity (Tay et al., 2001a), so did a short cycle time (Tay et al., 2002c).

As so many factors can affect the granulation process, it seems too early to draw a conclusion as to exactly how granular sludge is formed under aerobic conditions. However, it is generally agreed that the formation of aerobic granules is not a random phenomenon of microbial adhesion, but a directive process triggered or enhanced by

certain conditions. It is a gradual process from suspended flocs to compact aggregates, further to granular sludge, and finally mature aerobic granules (Tay et al., 2001a), although some of the characteristics of the sludge may exhibit sudden changes during the granulation process (Liu et al., 2003).

Liu et al. (2005c) reviewed the factors involved in aerobic granulation in SBR, and identified the major selection pressure as the settling time and exchange ratio, both of which favor the wash out of slow-settling flocs, and “force” the cells to aggregate. Other factors such as shear force, feast-famine regime, pulse feeding, higher reactor height to diameter ratio and proper solid and hydraulic retention time can all contribute to the “selection pressure” inducing aerobic granulation. The formation of aerobic granules is believed to be a result of high shear force, short hydraulic retention time, selection pressure against slow settling particles and frequent shift between feast and famine (Beun et al., 1999; Liu et al., 2005c).

2.5.2 Characteristics of aerobic granules

Granular sludge is most remarkable in its good settling ability, which is possibly due to the larger size, although its density is not necessarily much higher than that of activated sludge (Guiot et al., 1992). Etterer and Wilderer (2001) concluded that the wet density of aerobic granules did not deviate significantly from activated sludge flocs, after comparing this parameter of the two kinds of biomass ($1.044\sim1.048\text{ g mL}^{-1}$ for aerobic granules, $1.036\sim1.062\text{ g mL}^{-1}$ for flocs). However, the settling ability of granules is significantly higher than flocs. The SVI (sludge volume index) of activated sludge may be in the range of $100\sim200\text{ mL g}^{-1}$ (McSwain et al., 2005), while those of granules can be as low as 30 mL g^{-1} (Moy et al., 2002; Tay et al., 2002a). At the end of SBR cycles, big and dense granules could settle quickly, therefore only a short settling time is needed (minutes compared to hours for activated sludge in SBR), and the operation efficiency of the reactor is enhanced. Moreover, the fast settling of granules enables the retention of large quantity of biomass within the reactor, thus improves the removal of substrates.

The retention of high concentration of biomass not only enables granules to efficiently degrade the target substances in the wastewater, thus stand high organic loading, but also improves the tolerance of the reactor towards toxic compounds, e.g. phenol. The toxicity of substances like phenol is much dependent on its concentration, as introduced in section 2.3. At high concentration, phenol inhibits the degradation and metabolic activities of bacteria. When microbial ability of phenol degradation is inhibited by its concentration, phenol accumulation occurs and the toxicity sustains. However, when large quantity of biomass is present in the reactor, the high phenol concentration can be quickly reduced to non-toxic level, even though the specific phenol degradation activity of the biomass might not be high. Aerobic granules have been successfully cultivated on phenol, with a mean size of 0.52 mm and a sludge volume index of 40 mL g SS⁻¹. These granules exhibited outstanding ability to tolerate high phenol concentration and loading rate, as well as to degrade phenol at a remarkable rate, i.e. the specific oxygen utilization rate with phenol reached 110 mg O₂ g VSS⁻¹ h⁻¹, and the kinetic parameters obtained indicated an actual maximum phenol degradation rate of 1.4 g phenol g VSS⁻¹ d⁻¹, at a corresponding phenol concentration of 320 mg L⁻¹ (Jiang et al., 2002, 2004a; Tay et al., 2004b).

Granular sludge is more resistant to toxic compounds than flocs of activated sludge also because it is a form of immobilized cells. Keweloh et al. (1989) found that the toxicity of phenol was significantly reduced when cells were immobilized in calcium alginate. Cells entrapped in hollow-fiber membranes (Loh et al., 2000) also exhibited tolerance to higher phenol concentrations than free cells. Therefore as a kind of aggregated cells, aerobic granules can be expected to have better protection from phenol toxicity compared to common activated sludge (Tay et al., 2004b).

One possible reason for the resistance to toxic substances is that diffusion limitation occurs in cell aggregates of big size and dense structure. While diffusion barrier might limit oxygen and nutrient transportation to the core of the aggregates, it can also protect the inner layer of microorganisms against exposure to high concentrations of toxic substances in the bulk liquid (Villaverde and Fernandez-Polanco 1999; Rittmann et al.,

2000). Liu et al. (2005a) found that nitrifying granules were resilient to the inhibitory effect exerted by phenol, indicating that the tolerance toward phenol toxicity is not only caused by its fast removal by biomass, but also by the granules' intrinsic structural advantage as cell aggregates.

Other forms of cell aggregates, i.e. biofilm and anaerobic granules have been extensively studied, but the research on granules formed under aerobic conditions didn't start until lately (Morgenroth et al., 1997). Apart from the aspects of the mechanism of granule formation and operational condition of granular sludge reactor, the application of aerobic granules is also of importance. One possible application of aerobic granular sludge and aerobic granular sludge blanket reactor is the co-metabolism of recalcitrant substances, e.g. TCE.

2.5.3 TCE degradation by immobilized cells

TCE degradation by mixed or pure culture biofilms has been conducted with methanotrophic consortia (Strandberg et al., 1989; Strand et al., 1991; Arvin, 1991), propane oxidizers (Phelps et al., 1990), *Burkholderia cepacia* G4 (Radway et al., 1998), *Burkholderia cepacia* PR123 (Sun & Wood, 1997) and *Methylosinus trichosporium* PP358 (Fitch et al., 1996). Attached cells tend to have much higher biomass densities than dispersed cells, and are able to have better biomass retention in one reactor (Anderson & McCarty, 1994); therefore may behave better in TCE toxicity tolerance.

2.5.4 Effect of EPS on TCE degradation

EPS—extracellular polymers are considered as a very important factor in granule formation. They act as a sticky, strong framework on which cells are attached and form aggregates (Tay et al., 2001b, Liu et al., 2004a). As EPS can accumulated on the cell surface, the surface characteristics of cells, e.g. hydrophobicity, surface charge density, binding site and surface morphology can also be affected (Liu et al., 2004a). Since the first step of TCE co-metabolism by cells is the intake of TCE across the cell membrane to

the enzymes, the surface characteristics can have significant effect on the binding, intake or repelling of TCE, hence increase or decrease the overall degradation rate.

Meanwhile, extracellular polymers can act as a barrier against toxic compounds, e.g. heavy metal ion, biocides and organic solvents (Flemming and Wingender, 2001) by sorption and dilution. Such phenomenon was observed by Ayala-del-Río (2002), that cells chronically exposed to TCE and phenol produced much more EPS than those exposed to phenol alone, and the morphology of the former showed fungi-like structure and more attachment of cells. It suggested that in order to respond to TCE-mediated stress, microorganisms might tend to form aggregates, and immobilized cells might have a higher tolerance to TCE toxicity, especially the solvent stress.

2.5.5 Effect of the diffusion limitation on TCE degradation

As in other forms of immobilized cells (e.g. biofilms and anaerobic granules), diffusion limitation occurs in aerobic granules (Beun et al., 2002; Li and Liu, 2005). Both the substrate and oxygen may become limited with the increasing depth of a granule (Wilen et al., 2004a), and the limitation is correlated with the composition of microbial functional groups in the granules (Wilen et al., 2004b). When inner layers of cells experience oxygen limitation, anaerobic degradation might occur (Etterer & Wilderer, 2001; Tay et al., 2002b). When substrate is depleted, the cells die and form inert biomass in the core of a granule, the accumulation of which eventually causes the granules to break up (Tay et al., 2002b).

The diffusion limitation may be either advantageous or disadvantageous to TCE degradation. The disadvantage is that it may cause resistance to TCE uptake by cells, therefore decrease TCE degradation rate. Indeed it has been observed that both the specific substrate removal and biomass growth rates were negatively correlated with the size of the granules (Liu et al., 2005b). However, as the toxic products from TCE degradation are diffusible, the limitation may also act as a protective barrier against toxicants, thus reduce cell inactivation.

2.5.6 Effect of storage granules on TCE degradation

Many bacteria grown under unbalanced conditions may be able to accumulate storage granules within the cells (Nichols & White, 1989). The unbalanced growth implies the growth stage when the substrate is in excess, and even if the bacteria grow with their maximum growth rate, the substrate is still not degraded fast enough. Some bacteria develop mechanisms to convert the excess substrate to polymers. Those polymers include glycogen, lipids or polyhydroxyalkanoates (PHA), and can be accumulated within the cells as storage granules. Poly- β -hydroxybutyrate (PHB) is the most dominant storage polymer as it is directly formed from the central metabolite acetyl-CoA (Doi, 1990).

A likely scenario where the unbalanced growth can occur is the sequencing batch operation mode (SBR mode). In a reactor operated under SBR mode, feast and famine periods exist in one operation cycle. In the feast stage, the substrate is in excess, and in the famine stage it is depleted. The repetitive shifting between substrate over-loading and starvation may trigger the formation of storage granules, which will service as an energy source for maintenance and growth during the famine stage. PHB storage was observed in aerobic granular sludge reactors, which is often operated in an SBR mode (Beun, 2001; Qin et al., 2005).

PHB storage granules were found to directly enhance TCE degradation, as a source of electron donors for the TCE degrading cells in the resting state (Henry and Grbic-Galic, 1991a; Henrysson and McCarty, 1993). The possible PHB storage in aerobic granules stimulated by the SBR mode of AGSB reactor might be a considerable advantage to sustain TCE co-metabolism by the granules in resting stage.

2.5.7 Effect of reactor type on TCE degradation

Two stage reactors have been proposed for the separation of TCE degradation and cell growth (Alvarez-Cohen & McCarty, 1991d). Alternatively, a reactor that is intermittently fed with TCE and the primary substrate can also serve the purpose, and is easier to operate. A sequencing batch mode reactor used in forming aerobic granules may be a

good example. By adjusting the operation mode to add in a TCE degradation phase, circumvention of competitive inhibition and the operation of cell recovery can be easily realized.

There is also a big problem encountered in two-stage reactor which can be easily solved in an aerobic granular sludge SBR. After exposure to TCE, cells in the transformation tank of a two-stage reactor may be inactivated to different degrees, so it's hard to decide whether to waste the biomass or to recover them. This problem can be overcome in an aerobic granular SBR. By applying a short settling period, dead cells will be washed out, or may be used as the material to build new granules. Automatic selection of active biomass can be achieved in an aerobic granular sludge reactor operating in the sequencing batch mode.

2.5.8 Possible advantages of AGSB reactor in TCE co-metabolism

❖ High biomass retention in the reactor

The biomass retention in the reactor has been proposed to be important for successful TCE co-metabolism (Anderson & McCarty, 1994). In an aerobic granular sludge blanket reactor (AGSB reactor), biomass concentration can be maintained as high as 7~8 g L⁻¹ (Jiang et al., 2004a). While in a sequencing biofilm reactor fed with phenol, a typical biofilm concentration of 2 g L⁻¹ was observed (Segar et al., 1995). Biomass concentration alone cannot assure successful TCE degradation, but the ability of AGBS reactor to retain high concentration of biomass for a long time is advantageous for a stable and sustainable TCE co-metabolic process.

❖ Tolerance to phenol toxicity

Jiang et al. (2002, 2004a) have demonstrated the tolerance of aerobic granules to high phenol concentrations and loadings. As mentioned in Section 2.3.3.5, phenol concentration applied to recover TCE-injured cells may be crucial to a successful degradation process. In order to recover the cells, phenol dosage should be high enough, but not so high as to cause toxic effect. As aerobic granules can tolerate high phenol

concentrations, the recovery potential of cells from TCE TPT can be greater for granules than for suspended cultures.

❖ Diffusion limitation

The toxicants from TCE degradation are diffusible, therefore when cells are protected by diffusion limitation, they could be less affected by the toxic effect. Therefore, aerobic granules may be more resistant to TCE TPT.

❖ Survival of starvation

AGSB reactors are usually operated in a sequencing batch mode. In every cycle, the starvation period can be as long as 2 to 3 hours, so aerobic granules are adapted to frequent starvations. TCE degradation can be conducted with resting cells to avoid competitive inhibition from the primary substrate. Therefore the ability to survive starvation may be important to a sustainable TCE co-metabolism process.

2.6 Summary

Fig. 2.8. Factors affecting TCE co-metabolism by aerobic granules fed on phenol.

In summary, the relationship of TCE, phenol and aerobic granules can be depicted as above. The three core constituents of co-metabolism are replaced by more specified factors: TCE—co-metabolic substrate; phenol—primary substrate and aerobic granules—microorganisms. Phenol and TCE are competitive inhibitors to each other, but phenol is essential in the induction of PH and in providing reducing energy, energy for cellular growth and recovery. Phenol itself is a toxic compound, but aerobic granules are fairly resistant to the toxicity. The same mechanism may also function in the resistance to TCE TPT and solvent stress. TCE exerts a profound impact on microbial community, which may be a combination of induction, inactivation, selection and other unknown factor(s).

CHAPTER THREE

FORMATION OF PHENOL-DEGRADING AEROBIC GRANULES AND THEIR TCE DEGRADATION ACTIVITIES CHANGE DURING GRANULATION PROCESS

3.1 Introduction

As introduced in the previous chapter, aerobic granule is a novel kind of autoaggregated cells. Aerobic granules have been successfully cultivated using toxic compounds such as phenol as the growth substrate (Jiang et al. 2002 & 2004a), and have shown the advantage of high degradation rate and tolerance to substrate toxicity. However, they have never been tested for the potential of co-metabolic degradation, such as that of TCE.

The high shear rate in the aerobic granular sludge blanket (AGSB) reactor gives granules a compact structure and a high density, and the diffusion limitation might occur when the diameter of the granules is large (Beun et al., 2002). Therefore, aerobic granules might be expected to have a lower specific TCE degradation activity than common phenol degrading activated sludge. However, the fast settling ability of the compact granules also enables high biomass retention in the reactor, and the lower specific TCE degradation activity might be compensated by the high biomass quantity.

The structure of the granules can also function as a diffusion barrier against toxic substances, such as the membrane-disturbing phenolic compounds (Keweloh et al., 1990). In the case of TCE co-metabolism, diffusive toxicants are produced and the turn-over dependent toxicity can inactivate cells. The diffusion limitation may then be the advantage of aerobic granules against the transformation product toxicity.

Therefore, aerobic granules may have a unique behavior in the co-metabolic degradation of TCE, which depends much on the interactions between and among the microorganisms, the growth-substrate and the co-substrate. However, the knowledge on the activities of aerobic granules in co-metabolism is lacking.

Therefore in this study, TCE was used to evaluate the potential application of aerobic granules in co-metabolic degradation. The specific TCE degradation activities of the phenol-grown aerobic granules could be compared to those of activated sludge for their evaluation. However, as the studies of TCE co-metabolism by other toluene or phenol grown sludge were done under different conditions, the results obtained in literature were not directly comparable. To obtain a fairer comparison, the TCE co-metabolic degradation activities can be traced through a granulation process, from the seed activated sludge to small granules and to mature granules. The changes of these activities may serve as an indication of the how fast and how much TCE can be degraded by phenol-grown aerobic granules.

Two parameters were chosen for the evaluation of TCE co-metabolic activities change during granulation, the specific instant transformation rate (T_r) and the ultimate transformation capacity (T_c). T_r is the instant rate of TCE degradation (how fast), and likely represents the enzyme activity of phenol hydroxylase towards TCE. T_c is the quantity of TCE that can be transformed per unit of biomass before it is total inactivated (how much). These two parameters are the most important in assessing the microbial activity in TCE co-metabolism and in designing relative applications.

In this study, phenol was used as the growth substrate to cultivate TCE degrading biomass. Phenol acclimated biomass was used as the seed sludge for the aerobic granulation. The T_r and the T_c of the sludge were monitored during the aerobic granulation process. The changes of both parameters and other biomass characteristics were monitored and evaluated.

3.2 Materials and methods

Chemicals and media

Phenol stock solution was made by directly weighing phenol crystal (Merck, Darmstadt, Germany) and dissolving it to give the intended concentration of 50 g L^{-1} . TCE stock solution was prepared by adding 2 mL of pure TCE (J.T. Baker Inc., Phillipsburg, NJ, USA.) to a 60 mL serum bottle which was pre-filled with MilliQ water and glass beads. The water was filled to the neck to eliminate headspace. The bottle was immediately sealed and vigorously shaken to thoroughly mix the content. Then the solution was kept in 25°C incubator overnight before using. As a clear partition of organic and water phase could be seen in the bottle, the solution was considered to be saturated with TCE at a concentration of 1100 mg L^{-1} (25°C).

Chemicals for reactor operation were of technical grades, and those for batch studies were of analytically grades. For batch tests, MilliQ water was used in all the procedure (USF ELGA, Chemoscience Pte. Ltd.), while tap water was used to make up the reactor influent.

The composition of the media for sludge acclimation and granules cultivation was as follows: phenol, NH_4Cl , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KH_2PO_4 , K_2HPO_4 and $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ at a weight ratio of 1: 1: 0.1: 0.2: 0.2: 0.05. Acetate was normalized to phenol concentration by converting to COD. The media would ensure that growth of biomass was only limited by the carbon source. The media were also supplemented with 1 mL L^{-1} trace elements solution as described by Futamata et al (2001a), the composition of which was: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.174 g L^{-1} ; H_3BO_3 , 0.232 g L^{-1} ; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.008 g L^{-1} ; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.008 g L^{-1} ; $(\text{NH}_4)_2\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.22 g L^{-1} ; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.096 g L^{-1} ; $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 0.116 g L^{-1} . The influent was adjusted to pH 7.5 by 1M NaHCO_3 .

For the batch studies, phosphate-buffered mineral media (Mu & Scow, 1994) were modified and supplemented with 5mL L^{-1} trace elements solution mentioned above.

Table 3.1. Composition of phosphate-buffered mineral media

Chemical	Concentration (g L ⁻¹)
KH ₂ PO ₄	0.694
K ₂ HPO ₄	0.854
(NH ₄) ₂ SO ₄	1.234
MgSO ₄ ·7H ₂ O	0.860
CaSO ₄ ·2H ₂ O	0.176
FeSO ₄ ·7H ₂ O	0.001

Thus, the media were eliminated of chloride ions, enabling the measurement of release of Cl⁻ as an indicator of complete mineralization of TCE.

Reactor design and operation

The configurations of the column reactors were as described by Jiang et al (2002), with 5 cm inner diameter and 2.0 L working volume. Reactors were housed in a temperature-controlled room at 25°C. The sequencing batch mode was operated with cycle time of 4 hours. The cycle time was divided into 4 periods: 220~222 min aeration, 3~15 min settling, 2 min effluent withdrawing and 3 min influent filling. Effluent was discharged at the port 60 cm above the reactor bottom, with the volumetric exchange ratio of 50%. The hydraulic retention time was 8 h and aeration rate was 3.5 L min⁻¹.

Air-stripping loss of phenol from the reactors

Phenol solutions of various concentrations were filled in the reactors to the volume of 2 L, and were aerated for 4 h at the rate of 3.5 L min⁻¹. The initial and final concentrations of phenol in the reactors were determined to estimate the loss of phenol due to air stripping. One concentration was tested with one column, and duplicated samples were taken for phenol concentration measurement..

Acclimation of activated sludge to phenol

The seed activated sludge was obtained from Jurong Waste Water Reclamation Plant and was then subject to increasing phenol concentration for acclimation. The characteristics of the seed sludge were as follow: MLSS, 3.8 g L^{-1} ; MLVSS, 3.4 g L^{-1} (88% of MLSS); SVI, $260 \text{ mL g TSS}^{-1}$ and floc size, $43.8 \mu\text{m}$.

The acclimation was carried out in two column type reactors described above with identical operation conditions: 220 min aeration, 15 min settling, 2 min effluent withdrawing and 3 min influent filling. Other conditions were the same as described above.

Two substrates were used in the feeding influent: phenol and sodium acetate. Sodium acetate was supplied as a secondary carbon source to support biomass growth, as it has been used as a non-competitive substrate in TCE co-metabolism by many researchers (Sun and Wood, 1996). In the course of acclimation, the concentration of sodium acetate was gradually decreased, and the influent phenol concentration was increased from 100 mg L^{-1} to 500 mg L^{-1} . Finally sodium acetate was eliminated from the influent and phenol was fed as the sole carbon and energy source, with the loading of $1.5 \text{ kg phenol m}^{-3} \text{ d}^{-1}$. At any time of the acclimation, the total COD loading was $3000 \text{ mg COD L}^{-1} \text{ d}^{-1}$.

During the acclimation, the phenol concentration in the effluent and the phenol degradation profile within the reactors were periodically monitored, and the tests were done in duplicates.

Granulation

After two months when the seed activated sludge was fully acclimated to the phenol concentration of 500 mg L^{-1} , the settling time was gradually decreased from 15 to 3 min. Granulation was conducted by applying a short settling time to wash out slow settling particles. During the granulation, the suspend solids (SS and VSS) of the reactors were regularly measured. Other characteristics of the sludge: sludge volume index (SVI),

specific oxygen utilization rate (SOUR) with 250 mg L⁻¹ phenol and sludge size were also monitored. TCE degradation ability was indicated by two parameters: the instant TCE transformation rate (T_r) and the ultimate transformation capacity (T_c).

Sludge characterization

The SS, VSS, SVI and SOUR measurements were done according to APHA (1998) standard methods. Total solids were quantified by filtrating duplicated samples through 1 μ m pore size glass fiber filters and drying at 105 °C overnight. By further heating at 550°C for 20 min, MLVSS values were obtained. Sludge volume index (SVI) was measured through free settling tests. 100 mL liquid from the reactor was allowed to settle in a cylinder for 30 min, and the volume of solid phase was divided by the MLSS to obtain SVI value. SVI test was not duplicated for one reactor.

The biomass particle size was measured using a laser particle size analysis system (Malvern Mastersizer 2600), when sizes were smaller than 500 μ m. An image analysis system (Quantimet 500 image analyzer, Leica Cambridge Instruments) was used to measure particle size higher than 500 μ m in diameter. All the tests were done in duplicates. Sludge size and morphology can be viewed as an indicator of the granulation process.

SOUR (specific oxygen uptake rate) was tested by incubating known amount of biomass with phenol and measuring the dissolved oxygen concentration at time intervals of 15 s for 15 min or until DO became limiting. The oxygen consumption rate was divided by VSS to give SOUR. The phenol concentration applied in SOUR test was the same as the initial concentration in the reactor at the beginning of every cycle. One sample was obtained for the sludge from one reactor.

TCE degradation activities

The sludge was harvested from the reactor regularly, washed with modified phosphate-buffered mineral media (Mu & Scow, 1994) supplemented with 5 mL L⁻¹ trace element described above, and resuspended in the same media. 10~20 mL of the washed sludge was dispensed in a 60 mL serum bottle, giving the biomass quantity of 30~50 mg SS in every bottle. The bottles were sealed with Teflon septa and aluminum crimps, and 100 μ L of TCE saturated stock solution (1100 mg L⁻¹) was injected to every bottle through a gas tight syringe (SGE). Duplicated samples were tested for the sludge from one reactor.

TCE is a volatile compound, and could partition between the liquid and gaseous phases. In this study, 20 mL liquid and 40 mL gaseous phase were provided in each test vial. Therefore, to account for the partitioning, a dimensionless Henry's law constant of 0.39 (Gossett, 1987) was used. The initial concentration of TCE in each bottle was calculated to be 3 mg L⁻¹ in the liquid phase and approximately 1.2 mg L⁻¹ in the headspace. The instant TCE transformation rate of the sludge was determined by measuring the decrease of TCE mass in the bottle over a period of 3 h, at 10~20 min interval.

TCE was repeatedly injected to the bottle until no more decrease of TCE concentration was observed. As the test period could be as long as 7 days, every 24 to 36 h, the bottles were opened and the air was pumped into the headspace to replenish oxygen. The 40 mL headspace in the serum bottles was used as the source of oxygen for TCE degradation between the replenishment. The amount of O₂ in the headspace was calculated to be around 12 mg, while total degradation of 1 mg TCE required around 0.8 mg O₂. Assume an average endogenous respiration rate of 10 mg O₂ g VSS⁻¹ h⁻¹ for the fresh sludge, and a VSS content of 30 mg per bottle, the oxygen consumed by biomass endogenous respiration in 24 h would be around 7 mg. As maximum 1 mL TCE was injected everytime, and the replenishment interval was 24~36 h, the oxygen contained in one serum bottle was sufficient to sustain TCE degradation within this period. Before and after the bottles were opened, the TCE concentration in the bottle was measured. The transformation capacity was calculated as the total mass of TCE degraded divided by the total mass of sludge in the bottle.

INT staining

INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride) is a tetrazolium salt used as an indicator of cellular respiratory activities. When reduced, it forms dark red deposit intracellularly which could be observed with light microscope. The quantity of the deposit and therefore the intensity of the red colour are in proportion to the respiratory activity of the cell. The granules were incubated with 0.2% INT solution for 10 min, and subsequently fixed using tissue freezing medium (JUNG) and sliced to 20 μm thin layers with Cytomicrotome (Leica CM 3050S). The sliced granules layers were observed under light microscope (Olympus BX60) to see the granule structures.

Analytical methods

TCE: A Hewlett Packard 5890 gas chromatography equipped with a DB-624 column (J&W) and an FID was used to measure the TCE concentration. The conditions were as follows: carrier gas, helium; flow rate, 2.30 mL min^{-1} ; injection temperature, 200°C; detector temperature, 200°C; oven temperature isothermally at 150°C. The standard curve was obtained using the same medium, TCE stock solution and serum bottles, with a detection limit of 0.05 mg L^{-1} in liquid and the linear range up to 40 mg L^{-1} in the liquid. The standard curve is shown in Fig. 3.1, with an $R^2=0.9996$.

Fig. 3.1. TCE standard curve. The trendline shows the result of linear regression on the average of triplicated samples.

TCE concentration was determined by injecting 50 μL of the bottle's headspace content into GC. The leakage and adsorption by test materials and dead sludge (HgCl_2 treated) were also measured. Tests were performed in triplicates.

All the tests were done at room temperature of 23~25°C.

Phenol: The modified colorimetric method of Folsom et al. (1990) was adopted for direct phenol quantification. 50 μL NH_4OH (2 N) and 25 μL 4-aminoantipyrine solution (20 g L^{-1} , Osaka Hayashi Pure Chemical Industries Ltd. Japan) was placed in a 1.5 mL centrifuge tube, and 1 mL of sample was added and mixed. 25 μL of $\text{K}_3\text{Fe}(\text{CN})_6$ (80 g L^{-1} , Merck) was further added and the absorbance at 500 nm was measured using a 1 cm cell and a spectrophotometer (SHIMADSU, UV-1201V). The concentration of phenol was obtained by a standard curve with medium as the blank. The detection limit was 0.1 mg L^{-1} and linear range up to 20 mg L^{-1} . The correlation was 0.9995 with triplicated samples.

3.3 Results

3.3.1 Preliminary tests

3.3.1.1 Air stripping lost of phenol from reactors

Because a high aeration rate (3.5 L min^{-1} per reactor) was used for the granulation process in this study, it was necessary to determine the effect of air stripping on the loss of phenol mass from the reactors. 2 L tap water containing phenol of different concentrations was aerated for 4 h (duration of one cycle) and the phenol concentrations were measured before and after the aeration. Table 3.2 shows the result. No significant loss of phenol was observed, and some results showed negative loss (increase of phenol concentration after aeration). This could be due to the evaporation of water from the reactor. Therefore, decrease of phenol concentration could be entirely attributed to biomass activities in the later reactor operations.

Table 3.2. Loss of phenol from the reactor due to air stripping from duplicated samples

reactor no.	R1	R2	R3	R4
intended phenol concentration (mg L ⁻¹)	50	100	200	400
initial phenol concentration (mg L ⁻¹)	37.1	85.5	204.4	395.9
final phenol concentration (mg L ⁻¹)	41.5	85.9	182.9	401.9
loss of phenol (%)	-11.8	-0.40	10.5	-1.5
average loss (%)		-0.8		

3.3.1.2 Acclimation of activated sludge to phenol

Phenol is a toxic substance which could inhibit microbial growth or even kill cells (Yap et al., 1999). To conduct aerobic granulation with phenol as the sole carbon and energy source, the seed sludge was first subject to increasing phenol concentrations for acclimation. The acclimation was done in the same column type reactors as were used for granulation, and the influent phenol concentration was increased from 100 to 500 mg L⁻¹ in two months, during which sodium acetate was used as a complementary carbon and energy source. The combination of the two substrates gave a total COD loading of 3000 mg L⁻¹ d⁻¹. The change of concentrations of phenol and acetate is shown in Fig. 3.2, and Fig. 3.3 shows the change of SS and phenol-dependent SOUR during acclimation.

Fig. 3.2. Change of influent phenol and sodium acetate concentrations in acclimation period. (■) phenol (▲) sodium acetate.

Fig. 3.3. Change of the suspended solids (SS) and the specific oxygen utilization rate (SOUR) with phenol during acclimation, with duplicated samples. (■) SS (▲) SOUR.

Because a rather short settling time (15 min) was applied, a large proportion of the seed sludge was washed out in the first week, and the SS showed a decreasing trend. However, it didn't affect the overall phenol degrading activities of the reactors, as the phenol concentrations in the effluent were essentially below detection limit (data not shown).

This could be due to the gradually increased phenol loading and the largely improved phenol-dependent SOUR (especially after influent phenol concentration reached 500 mg L⁻¹). It indicated the acclimation of the seed activated sludge to phenol, and the induction of enzymes on the phenol degradation pathways. Another phenomenon to illustrate the acclimation process is the phenol degradation profile during a cycle. A sample of one cycle is shown below in Fig. 3.4.

The cycle was measured on day 45, and the initial phenol concentration was 207 mg L⁻¹. The activated sludge in the reactor was able to degrade phenol to below detection limit within 2 h, but an accumulation of non-phenol organic carbon was observed from 1 to 4 hours of the cycle. It coincided with a yellow color observed 15 min after the cycle started, which might be caused by the accumulation of meta-cleavage intermediates (mainly 2-hydroxymuconic semialdehyde). However, as the acclimation proceeded, phenol degrading ability was significantly improved. When a cycle was again measured at day 60, the initial 268 mg L⁻¹ phenol was degraded within 45 min, and the accumulation of non-phenol organic carbon and yellow color were no longer observed.

Fig. 3.4. The phenol and non-phenol organic carbon concentrations change during one degradation cycle. The test was with duplicated samples. (■) non-phenol organic carbon (▲) phenol.

3.3.1.3 Degradation test of TCE by acclimated phenol degrading sludge

At day 65 of the acclimation, the activated phenol-degrading sludge was harvested and sealed in 60 mL serum bottles for TCE co-metabolism test. Various amounts of TCE were injected to the bottles to obtain the intended concentrations. The bottles were then vigorously shaken by hand for 15 s and the initial headspace concentrations were measured. The reaction was monitored for 5 h at 30~60 min intervals. No exogenous reducing energy source was added to the reaction bottles, because it was not intended to be added in real operation. Test was done in duplicate and a bottle without biomass was used as the blank to correct for the adsorption and volatile loss. The result is shown in Fig. 3.5.

The approximate 63 mg of phenol-grown sludge in every test vial was able to degrade 0.4 mg L⁻¹ TCE in the liquid in 1 h, and 2.95 mg L⁻¹ in 3 h. When the test was extended to 24 h, the vial with the initial TCE concentration of 14.9 mg L⁻¹ was also found to be cleared of TCE. The control vial (8.6 mg L⁻¹ TCE in the liquid) showed no significant loss of

TCE. Therefore, the phenol-acclimated activated sludge was indeed able to degrade TCE to below detection limit.

Fig. 3.5. TCE co-metabolic degradation by phenol-acclimated sludge. (■) blank (8.64 mg L⁻¹), (▲) 0.40 mg L⁻¹, (●) 2.95 mg L⁻¹, (×) 14.9 mg L⁻¹; except the blank, the other test vials were incubated with 3.14 g L⁻¹ sludge and the samples were not duplicated.

3.3.1.4 Abiotic loss of phenol in the test flasks in the kinetic studies

Kinetic studies of phenol degradation were carried out in the open-mouthed shake flasks, and mixing was provided by aeration, therefore the loss of phenol due to air-stripping and adsorption to test materials and biomass was first determined.

Phenol solutions of different concentrations were aerated either without biomass (air-stripping loss) or with HgCl₂ killed aerobic granules (adsorption) in single samples. The initial concentrations of phenol were identical with the concentrations that would be used in kinetic studies, and the test durations and the sampling intervals were also kept the same. The test flasks were periodically sampled for phenol concentration analysis. The result is shown in Table 3.3.

Table 3.3. Change of phenol concentration due to air stripping and adsorption to dead biomass

initial conc. (mg L ⁻¹)	remained conc. (%)	sampling time (min)	0	5	10	20	40	60	120	180
5			100	98.2	--	--	--	--	--	--
10			100	100.1	104.5	--	--	--	--	--
50			100	99.2	100.5	--	--	--	--	--
100			100	97.2	96.2	97.7	--	--	--	--
500			100	--	--	97.7	97.7	101.1	--	--
1000			100	--	--	--	--	99.1	96.0	101.2

It could be seen that the test conditions used didn't cause significant drop of phenol concentrations. Therefore the air-stripping and adsorption to test materials were not the major sink on phenol mass in the test flasks. In the subsequent studies, decrease of phenol concentrations could be solely attributed to biomass activities.

3.3.1.5 Abiotic loss of TCE from the reaction vials

Factors other than microbial degradation could cause loss of TCE mass from reaction vials. Those factors included possible leakage, sorption to glass serum bottle, sorption to Teflon septa and sorption to biomass. Reaction vials containing HgCl₂ killed granules were tested for the TCE loss to assess the effects of those factors, and the result is shown in Fig. 3.6. For TCE of three concentrations, no significant loss was detected with the test materials and conditions. Therefore the abiotic loss of TCE from the test vials could be considered as negligible.

Fig. 3.6. Abiotic loss of TCE from reaction vials. (■) blank (0.22 mg L^{-1}), (▲) 0.21 mg L^{-1} , (×) 2.9 mg L^{-1} , (●) 12.2 mg L^{-1} ; except the blank, the other test vials were incubated with 2.82 g L^{-1} HgCl_2 killed granules with single samples.

3.3.2 Aerobic granulation using phenol as the growth substrate

The aerobic granulation process was carried out for more than 4 months using phenol as the sole carbon and energy source and the acclimated activated sludge as the seed. The settling time was gradually decreased from 15 min to 3 min to facilitate the washout of slow settling flocs and the retaining of fast settling granules. Changes of suspended solids (SS), sludge volume index (SVI), biomass size, phenol-dependent specific oxygen utilization rate (phenol-SOUR) and the T_r and T_C of TCE were traced during the granulation process.

The changes of the settling time, the concentration of suspended solids (SS) in the reactor, the mean size and the SVI of the sludge are shown in the Figs. 3.7 and 3.8. According to the SS concentration, biomass characteristics and reactor performance, the whole granulation process could be divided to four phases: the adaptation, pregranulation, granulation and stabilization phases.

Fig. 3.7. Change of settling time and MLSS concentration in the reactors during granulation. (■) settling time; (▲) mixed liquor suspended solids (MLSS), duplicated samples from two reactors.

Fig. 3.8. Change of biomass SVI (sludge volume index) and mean size during granulation. SVI was obtained from single sample for one reactor, and size was by duplicated measurements for the sludge from one reactors. (■) SVI; (▲) mean size.

In the first 6 weeks of the operation, the SS concentration in the reactor increased from 3 g L⁻¹ to 7~8 g L⁻¹ and SVI value dropped sharply. A concomitant rise of mean biomass size was not observed until the 8th week, as could be seen from Fig. 3.8. The characteristics of the reactor performance of this period were the accumulation of small and fast settling sludge, and it was termed the adaptation phase.

After the reduction of the settling time to 5 min, the biomass began to be washed out and the concentration of SS dropped down to 1~2 g L⁻¹. Contrary to the first phase, an increase of biomass size to approximately 300 µm could be observed during this stage. The SVI value showed a general decreasing trend after it reached 50 mL g SS⁻¹, with a periodical fluctuation between 30 and 50. The biomass in the reactor was a mixture of small granules and pinpoint flocs, the majority of which was the latter. This phase could be termed as the pre-granulation phase.

When the settling time was further decreased to 3 min at day 80, a sharp increase of mean biomass size was observed. The SS concentration also began to rise after a continuous decline of 10 weeks. SVI value further dropped at this stage to less than 30 mL g SS⁻¹. The SS, mean size of the biomass and the SVI exhibited minor fluctuation, but remained relatively stable in the next 4 weeks. These were the granulation and stabilization phases.

3.3.3 Phenol degradation activity change during granulation

Phenol degradation activity of the sludge was indicated by the phenol dependent specific oxygen utilization rate (SOUR), as shown in Fig. 3.9. In the first stage, the biomass exhibited a high SOUR value of 300~400 mg DO g VSS⁻¹ h⁻¹, followed by a drop to below 200. Such a decrease was concomitant with the shortening of settling time to 6 min and the SS concentration drop. In the pregranulation stage, SOUR exhibited a general increasing trend, and reached the highest value of 600 mg DO g VSS⁻¹ h⁻¹ at week 12. When granules were being formed, the SOUR value sharply dropped to 100~200, and subsequently stabilized. The total oxygen utilization rate (TOUR) showed a similar but

less fluctuating trend. After a major increase to $2300 \text{ mg DO h}^{-1} \text{ L}^{-1}$ and a subsequent decrease to 600 before day 56, the TOUR value stabilized around 800.

Fig. 3.9. Change of specific oxygen utilization rate (SOUR) and total oxygen utilization rate (TOUR) of the biomass during granulation. (■) SOUR; (▲) TOUR. Tests were done in duplicates.

3.3.4 Change of TCE transformation activities during granulation

TCE degradation activities were indicated by two parameters, the instant transformation rate (T_r) and the ultimate transformation capacity (T_c). An example of the T_r and T_c measurement is shown in Fig. 3.10.

Figure 3.10a shows the test of T_r , where the biomass was subject to TCE degradation instantly after the harvest from the reactors. The 54 mg phenol-grown biomass in each test vial was able to completely degrade 3 mg L^{-1} TCE within 2.5 h. Figure 3.10b shows the longer term of TCE degradation. When the concentration of TCE finally stopped decreasing after 60 h of test, the total mass of TCE transformed was divided by the total biomass in the test vial to give the transformation capacity.

Fig. 3.10a. Test of TCE transformation rate by phenol-grown biomass. (■) test vial 1; (▲) vial 2; (×) vial 3; (●) vial 4.

Fig. 3.10b. Test of TCE transformation capacity by phenol-grown biomass. (■) test vial 1; (▲) vial 2; (×) vial 3; (●) vial 4.

Fig. 3.11. Change of TCE transformation rate (T_r) and transformation capacity (T_c) during granulation. Data were obtained with duplicated samples from each reactor. (■) T_r ; (▲) T_c .

Both the parameters of T_c and T_r showed general decreasing trends during granulation (Fig. 3.11). The T_r of the seed sludge was $1.1 \text{ mg TCE g SS}^{-1} \text{ h}^{-1}$. It exhibited a fluctuation between 0.7 to 1.7 in the adaptation and pregranulation phases. When granules were formed, an increase and stabilization of T_r was observed. The transformation capacity of the seed sludge was $0.03 \text{ mg TCE mg SS}^{-1}$, which rose to above 0.05 in two weeks of operation. The high T_c was maintained for 5 weeks, followed by a reduction and minor fluctuations in the subsequent stages. The transformation capacity of phenol grown aerobic granules stabilized between 0.013 and 0.017 mg TCE mg SS^{-1} after day 95.

3.4 Discussion

3.4.1 Cultivation of aerobic granules

In this study, the granulation process and the change of biomass activity towards TCE in the process were monitored for more than 4 months. The scanning electron microscopy and INT-stained images of the stable aerobic granules formed are shown in Fig. 3.12 as the indication of their morphology and respiratory activity.

Fig. 3.12. Scanning electron microscopy images and INT stained light microscopy images of phenol-grown aerobic granules.

The SEM pictures show a spherical and compact granule, with bacteria of diverse morphologies attached on the surface. No obvious filamentous structure was observed. From the sliced and INT stained images the inner structures of the granule can be observed. There seemed to be a highly active layer of cells at the surface (as shown by the dark red and solid color), and loose and porous structures at the centre. It could be

postulated that the surface layer of cells carry the responsibility of degrading the substrates, and the inner net-like substances maintains the granule structure stability.

During the first 6 weeks of the granulation operation, a high SS concentration was observed, presumably due to the high settling time (15 min) applied at this stage, as well as the decrease of SVI from over 300 to less than 40 mL g SS⁻¹ within the first two weeks. The rapid improvement of sludge settling ability and the relative long settling time resulted in the good retention of biomass within the reactor, which might be advantageous when a toxic compound was used as the sole carbon and energy source, such as phenol. Therefore this phase could be termed as the adaptation phase.

In the pregranulation phase, the biomass size didn't exhibit a concomitant increase with the SS concentration. However, the decrease of biomass size didn't negatively affect the settling ability, and it might also be a reason for the high SS retention in the reactor during the adaptation phase.

The SS concentration exhibited a continuous drop from day 39 to day 80, during which the settling time was reduced from 6 to 3 min. It indicated the excessive washing out of slow-settling flocs due to the short settling period. Meanwhile, a steady increasing trend was observed for the mean biomass size in the reactor, indicating the gradual growth of small granules, which could be the precursors for stable aerobic granules.

A rapid development of granules was observed from day 80, the most obvious sign of which was the sharp increase of mean biomass size. The decreasing trend of SS in the reactor was reversed, presumably due to the formation of granules and retention of biomass in the reactor. The SS values after day 138 are further shown in Fig. 3.7, which denoted stabilization of biomass concentrations in the reactors. It seemed that before the settling time was reduced to 3 min, the reactor was in an unstable and critical state, and the change of operation condition triggered the rapid formation of large quantity of aerobic granules, by exerting a greater selection pressure.

3.4.2 Change of phenol and TCE degradation activities

The SOUR value of the biomass (as shown in Fig. 3.9) was measured with 250 mg L⁻¹ phenol, which was the same as the initial phenol concentration in the reactor at the beginning of every cycle. High SS concentration and the high SOUR were simultaneously observed in the first 6 weeks of operation. However, the phenomenon didn't sustain, probably due to the limited phenol supply and the decrease of settling time. Before the granules were formed, the SS in the reactor continued to drop, and the SOUR exhibited a general increasing trend. The SOUR once reached an highest value of around 600 mg DO g VSS⁻¹ h⁻¹, corresponding to the lowest SS of 1.74 g L⁻¹ in the granulation process, and the stabilized granules (SS 4~6 g L⁻¹) had an SOUR value of 100~200 mg DO g VSS⁻¹ h⁻¹. Therefore, except for in the early stage of operation, the SOUR and the SS exhibited a negative correlation, resulting in the relatively stable TOUR (total oxygen utilization rate in the reactors) from week 6. This was in accordance with the low effluent phenol concentration, which was constantly below detection limit.

The activity of biomass towards TCE could be indicated by the instant specific TCE transformation rate (T_r) and the ultimate TCE transformation capacity (T_C). The former indicates how fast can TCE be transformed by fresh biomass, while the latter represent how much TCE can be transformed before the biomass is totally inactivated. The change of TCE degradation activities of the biomass during granulation was monitored. As the inoculum used in the reactor was fully acclimated phenol-degrading activated sludge, changes observed for T_C and T_r could be attributed to the granulation process alone.

In the adaptation, granulation and stabilization phases, the two parameters show parallel changes. That is, when the biomass had high transformation rate, it exhibited high transformation capacity. However, in the pregranulation phase, the T_r continued to drop, while the T_C was maintained at a high level of 0.05~0.06 mg TCE mg SS⁻¹. After day 56, the trend reversed. The T_r exhibited a sharp increase and reached its highest level during granulation (1.7 mg TCE g SS⁻¹ h⁻¹), but the T_C began to drop.

The unusual changes of T_r and T_C may be due to the transient state of the pregranulation phase, when flocs and granules competed with each other, and the overall biomass activities fluctuated. The declining T_C after day 56 may suggest the occurrence of diffusion limitation, as an increase of biomass mean size was observed from day 53 on (Fig. 3.8).

The initial T_C and T_r for the seed sludge were $0.03 \text{ mg TCE mg SS}^{-1}$ and $1.1 \text{ mg TCE g SS}^{-1} \text{ h}^{-1}$ ($0.026 \text{ mg TCE mg SS}^{-1} \text{ d}^{-1}$), respectively. Among the limited studies on TCE co-metabolism by mixed culture phenol degraders, Chang & Alvarez-Cohen (1995b) reported a T_C and $V_{\max}$ of $0.03 \text{ mg TCE mg SS}^{-1}$ and $0.2 \text{ mg TCE mg dry cells}^{-1} \text{ d}^{-1}$. Therefore, the seed sludge in the study exhibited a comparable T_C but lower transformation rate for TCE. The reason of the lower T_r in this study could partly be due to the lower TCE concentration used in T_r measurement (approximately 3 mg L^{-1} in the liquid), but more likely was caused by the intrinsic lower enzyme activity of the seed sludge.

Both T_C and T_r decreased with the process of granulation. The stabilized granules had a T_r value of $0.8\sim 1.0 \text{ mg TCE g SS}^{-1} \text{ h}^{-1}$. Therefore the stabilized granular sludge was able to retain most of the instant TCE transformation rate of the seed sludge, although the size was 6 times higher for the granules.

However, the T_C , after a major rise from 0.03 to $0.05 \text{ mg TCE mg SS}^{-1}$, decreased when the granules were formed, and stabilized at around $0.015 \text{ mg TCE mg SS}^{-1}$. In comparison to the T_r change, stabilized phenol-degrading granules retained only half the initial TCE transformation capacity, probably due to the large biomass size and the compact structure of aerobic granules, causing diffusion limitation for phenol, oxygen and TCE (Beun et al., 2002, Tay et al., 2002b).

3.4.3 Correlation of phenol dependent SOUR and TCE T_r

SOUR was measured as the instant phenol-dependent oxygen consumption rate, and T_r indicated the instant TCE degradation rate. As both the first steps of phenol oxidation (phenol to catechol; Dagley, 1985) and aerobic TCE co-metabolism (TCE to TCE epoxide; Harker & Kim, 1990) are catalyzed by phenol hydroxylase, and both are the rate limiting steps in the degradation of the relative substrate (Semprini, 1997; Shingler et al., 1989), SOUR and T_r should change in the same trend.

Fig. 3.13. The change of dimensionless phenol SOUR to TCE transformation rate ratio with granulation.

The phenol dependent SOUR of the sludge was divided by its TCE T_r to obtain an dimensionless SOUR/ T_r ratio. The ratio of SOUR to T_r (Fig. 3.13) showed an fluctuation around 300 in the first 70 days of operation, indicating that the changes in SOUR and T_r were in parallel. However, from day 80 when granulation started, the ratio began to increase. After it reached 835 at day 100, a drop and subsequent stabilization were observed. Therefore, in this granule formation stage, the increase of SOUR outpaced that of T_r . In contrast, in the subsequent granule stabilization stage, the decrease of SOUR

was larger than the decrease of T_r . At the end of operation, the SOUR to T_r ratio (284.6) was slightly lower than that in the beginning (334.4). Therefore, except for the granulation stage, the SOUR and T_r changes followed the same trend throughout the entire operation; while during the formation and stabilization of granular sludge, the biomass activity towards phenol exhibited a greater fluctuation than its activity towards TCE.

The SOUR of the stabilized granules was 1/2 to 1/3 of that of the seed sludge, while T_r was hardly lost. These phenomena could be due to the changes of biomass characteristics when granules are formed. In this study, as well as many others (Futamata et al., 2001ab), the biomass activity towards phenol and TCE was measured as the whole cell activity, instead of the enzyme activity, due to the sensitivity of phenol hydroxylase to the enzyme purification process (Hino et al., 1998). Therefore, factors other than the enzyme activity, for example, the intake of the substrate by cells, might affect the overall substrate degradation rate, as suggested by Sun & Wood (1996). Phenol, having a hydroxyl group, is more hydrophilic than TCE, which is a slightly polarized and hydrophobic molecule. Liu et al. (2003) has shown the cell surface hydrophobicity increased when aerobic granules were being formed. Therefore, when biomass transforms from flocs to granules, the cell hydrophobicity increases, and the affinity of cells to TCE might increase as well. This might explain why the decrease of SOUR was greater than the decrease of T_r throughout the whole granulation process.

3.5 Conclusions

In this chapter, the results obtained in the preliminary study and the aerobic granulation process were presented.

The abiotic loss of phenol or TCE due to aeration and adsorption, whether in reactor operation or in batch studies was negligible. Therefore, microbial degradation was the main cause of phenol or TCE mass removal.

After acclimation to phenol for 2 months, the activated sludge exhibited enhanced phenol-dependent SOUR, which suggested the induction of relative enzymes and acclimation to high concentration of phenol. As phenol hydroxylase is the first enzyme on the pathways of phenol degradation, and also is the enzyme responsible for TCE co-metabolism, the acclimated sludge was found to co-metabolize TCE with considerable efficiency.

To evaluate the ability of phenol-grown aerobic granules in TCE co-metabolism, the acclimated sludge was further used to form aerobic granules with phenol as the growth substrate. The two important parameters in TCE co-metabolic degradation, the transformation rate (T_r) and the transformation capacity (T_C), were monitored throughout the granulation process.

After 4 months of operation, aerobic granules were formed and stabilized. Overall, the mean size increased from 50 μm to 600 μm , and the SVI dropped from 320 to 30 mL g SS^{-1} . The specific phenol degradation activity (SOUR) and TCE transformation activity (T_r) were almost retained for stable phenol-grown aerobic granules, but TCE transformation capacity was half lost, presumably due to diffusion limitation.

In summary, aerobic granules were successfully cultivated using phenol as the sole carbon and energy source in sequencing batch reactors, and have exhibited satisfying growth substrate and co-substrate degradation activities. Aerobic granules are able to degrade the target compound, TCE in sealed serum bottles, as fast as the phenol-grown activated sludge, although the size has increased by 12 folds. Though the apparent transformation capacity was reduced for stable aerobic granules, it can be compensated by the high biomass concentration in the AGSB reactor to achieve 100% TCE removal.

CHAPTER FOUR

CHARACTERIZATION OF TRICHLOROETHYLENE CO-METABOLIC ACTIVITIES OF PHENOL-GROWN AEROBIC GRANULES

4.1 Introduction

As introduced in Chapter Three, aerobic granules were formed using phenol as the growth substrate. The stable granules were subsequently tested for their TCE degradation activities. The parameters investigated were: the phenol and TCE degradation kinetics, the TCE transformation capacity, the mineralization potential, the dependence of T_r and T_c on exogenous electron addition and biomass size, and the effect of TCE direct and transformation product toxicity.

Kinetic parameters indicate the dependence of substrate degradation rate on the substrate concentration. Michaelis-Menton equation: $V = V_{\max} S / (K_S + S)$, is widely used to describe enzyme kinetics, and also is the one most often adopted for TCE co-metabolism. This equation indicates a first-order kinetic behavior when the substrate concentration S is low, and a constant degradation rate (zero-order) when S is high. K_S , the half saturation constant, is equal to the substrate concentration when the substrate degradation rate V is half the maximum rate $V_{\max}$. It also indicates the affinity of the enzyme to the substrate, and the lower K_S is, the higher the affinity will be. In most cases, TCE degradation kinetics are sufficiently described by Michaelis-Menton equation, but some studies reported a reduced TCE transformation rate under high TCE concentrations (Oldenhuis et al., 1991; Alvarez-Cohen & McCarty, 1991c; Hyman et al., 1995).

When the substrate transformation rate is reduced under higher concentrations, it is possible that the substrate exerts an inhibition effect on the enzyme. For instance, phenol is a well-known cell membrane disturbing substance, and its degradation kinetics exhibit a concentration-dependent inhibition under high phenol concentration. In such cases, the kinetics model should incorporate the inhibition effect. The Haldane's equation: $V = V_{\max}S/(S + K_S + S^2/K_I)$, is for such a purpose, and is commonly used for phenol degradation description (Bailey & Ollis, 1986). It also can be used in TCE degradation, as TCE is an organic solvent and might stress cells in contact with it in a mechanism similar to that of phenol.

Kinetic parameters are essential in understanding the microbial activity towards certain substrate, and also in designing bioreactors for relative applications, therefore they are first investigated in this chapter. The Michaelis-Menton kinetics were used to model TCE transformation, as no significant inhibition effect was observed in the preliminary TCE degradation test (refer to Chapter Three). The parameters to be determined are TCE $V_{\max}$ and K_S , the former of which indicates how fast TCE can be transformed, and the latter shows the affinity of phenol-hydroxylase towards TCE. Haldane's equation was adopted for phenol kinetics. K_I , the inhibition constant, indicates the severity of inhibition effect of phenol on biomass. The higher the K_I , the higher tolerance do cells have for phenol.

When TCE is totally mineralized, 3 mol of chloride ions should be released by 1 mol of TCE degraded. As the transform products are the non-toxic Cl^- , CO_2 and H_2O , TCE is totally detoxified. Therefore, the amount of Cl^- released can be viewed as an indication of TCE detoxification. The more chloride ions are released, the higher is the extent of TCE mineralization. The ideal result is the total mineralization and detoxification.

However, TCE transformation does not necessarily result in total mineralization. A serious problem of TCE aerobic co-metabolism is that it produces partially dechlorinated reactive substances which can inactivate cells that transform it. The toxic effect is defined as transformation product toxicity (TPT). The toxicant that inactivates cells is not TCE itself, but rather its transformation product(s). A simplified model (Alvarez-Cohen &

McCarty, 1991c) describes TCE TPT by the transformation capacity (T_C). T_C is the amount of TCE that could be degraded per unit of biomass before total inactivation. When the biomass transformed TCE, lowered cell viability (Heald & Jenkins, 1994), general respiratory activities (Chu & Alvarez-Cohen, 1999) and growth substrate degradation rates (Hyman et al., 1995) were observed. The more TCE is transformed, the lower the subsequent activities are. According to the model, when the cells have degraded TCE equal to their T_C level, the cells are totally inactivated, and the subsequent activities approach zero. In other words, the cells are considered as dead.

The transformation capacity is an important parameter in evaluating the microbial TCE degradation ability. Higher T_C indicates that cells can transform more TCE before they are totally inactivated, therefore are more resistant to TCE TPT.

Apart from the transportation product toxicity, TCE itself can have direct effect on the biomass. Organic solvents with a log K_{OW} (octane-water participation constant) between 1.5 and 3 may generally cause cytoplasmic membrane-associated toxicity (Sikkema et al., 1995). TCE has a log K_{OW} of 2.61, and could have a concentration-dependent harm on the cells when present in liquid phase.

TCE used to be viewed as a simply fortuitous co-substrate, and having no effect on the induction of the non-specific enzymes. However, TCE was found to induce the toluene oxygenases in various pure cultures (Heald and Jenkins, 1994; McClay et al., 1995; Leahy et al., 1996; Shingleton et al., 1998 and Park et al., 2002). Therefore TCE can induce its own degradation and the degradation of the growth-substrate, e.g. toluene, although not so efficiently as toluene itself.

In this chapter, the above mentioned parameters for TCE degradation activities of the stable phenol-grown aerobic granules were investigated. The aim was to gain the knowledge of how fast, how much and how thoroughly could the granules degrade TCE as a microbial mixed culture, and to compare the activities with those of other phenol-grown microbes.

4.2 Material and methods

Reagents and medium

The phenol stock solution, TCE stock solution and chloride free medium described in Chapter Three were used for the TCE degradation batch studies.

Preparation of granular biomass

Aerobic granules were harvested from stably operated aerobic granular sludge blanket (AGSB) reactors, centrifuged twice at 3000 g for 15 min, washed with the Cl⁻ free medium, and resuspended in the same medium to desired concentrations for batch studies.

Phenol kinetics

Aerobic granules were put into 250 mL shake flasks at the average concentration of 1.8 g SS L⁻¹ and the final volume of 50 mL. Different volumes of phenol stock solutions (50 g L⁻¹) were added to provide the desired final concentrations. Aeration was provided by air pumps and porous stones at the rate of approximately 1 L min⁻¹ per flask to mix the content and supply oxygen. At time intervals of 1 min to 1 h (depending on the initial phenol concentrations), triplicated liquid samples were taken and the phenol concentrations were analyzed. The initial rates of phenol removal were obtained by linear regression and plotted against the measured initial phenol concentrations. Non-linear regression was used to model the kinetic behavior using Sigma Plot 9.0 (Systat software, Inc.). Haldane's equation was adopted to describe the phenol degradation kinetics. The loss due to air stripping, sorption to test materials and biomass was measured as described in Chapter Three, and was found to be negligible.

TCE kinetics

After similar harvest and resuspension procedure as described above, phenol-grown aerobic granules were distributed to 60 mL serum bottles to the volume of 20 mL. The bottles were sealed with 47 mm Teflon faced septa (Wheaton) and aluminum crimps, with different amounts of TCE stock solution injected using gas tight syringes (SGE).

As TCE partitions between the liquid and gas phases, the determination of TCE degradation kinetics can be problematic. Many studies didn't account for the partitioning effect in a two-phase system, and TCE was treated as solely present in the liquid for convenience. Such a simplification can be misleading in two ways. First, depending on the relative volumes of the liquid and gaseous phases, the calculated TCE concentration in the liquid can be much higher than the real concentration, distorting the kinetic data. Second, TCE partitioned in the gaseous phase is not directly available to the biomass, therefore the transfer of TCE from the gas to the liquid phase could be rate-limiting.

A one-phase system (i.e. liquid phase only, no headspace) can solve the problems. However, in such a system, oxygen can be limiting, especially when the test duration is long and biomass concentration is high. Oxygen limitation can seriously affect TCE co-metabolism, as it is an aerobic process and catalyzed by oxygenases, which are sensitive to oxygen level (Ayoubi and Harker, 1998).

Considering all these factors, a two-phase system was adopted in this study. TCE partitioning between the two phases was accounted for, and TCE concentration was reported as the concentration in liquid. Transfer of TCE from the gas to the liquid phase was proven by previous studies not to be rate limiting, and all the TCE mass in the close system can be considered as available to the biomass (Folsom et al., 1990; Ayoubi & Harker, 1998). Therefore the rate of removal was calculated as the disappearance rate of total TCE mass.

TCE level in a two-phase close system can be indicated by the concentration in the liquid phase, in the gaseous phase, or the total concentration. The total concentration can be measured by organic solvent extraction, which dissolves TCE in both the liquid and the gas phases to the solvent phase. However, hexane was tested as the solvent at the volume ratio of 1:1 to the aqueous phase. The lower detection limit was higher than 5 mg L⁻¹ in the aqueous phase, and the sensitivity was not satisfying. Therefore, headspace concentration was chosen as a better indication of TCE level.

In a close system, the equilibrium of a volatile compound, e.g. TCE can be described by Henry's law, and a dimensionless Henry's law constant of 0.39 at 25°C (Gossett, 1987) was used for TCE. In other words, when TCE partitioning is in equilibrium in a sealed vial, the concentration in the gaseous phase (in unit of mg L^{-1}) is 0.39 of the concentration in liquid (also in mg L^{-1}) at 25°C. The total mass of TCE in such a vial is equal to $C_g V_g + C_l V_l$ (concentration in headspace $\times$ headspace volume + concentration in liquid $\times$ liquid volume).

For the measurement of TCE concentration, 50 μL headspace samples were drawn out by a gas tight syringe and injected to a gas chromatography. The obtained concentrations of TCE in the headspace were used to calculate the mass of TCE in the bottle. The specific rates of TCE mass removal were plotted against the initial TCE concentrations in the liquid, and the kinetics were modeled by Michaelis-Menton equation using non-linear regression. The abiotic loss of TCE due to possible leakage, sorption to bottles and biomass was also negligible. The tests were done in triplicates.

TCE transformation capacity

The transformation capacity (T_C) of TCE with resting cells (no phenol present) was measured by repetitively adding TCE to the test bottles containing aerobic granules. Every 24 to 48 h, triplicated bottles were opened and the headspace was purged with air to replenish oxygen. The TCE concentrations in the bottles were measured before opening and after re-sealing. The degradation was traced until the reaction totally stopped. The TCE mass transformed was divided by the quantity of biomass in the bottle to obtain T_C .

TCE mineralization potential

The mineralization potential was indicated by the Cl^- ion release. After TCE transformation, the supernatant of the reaction medium was analyzed for its chloride ion concentration in triplicates and against medium blank. The mass of Cl^- release was plotted against the correspondent mass of TCE transformed, and the slope obtained indicated how much chloride ion was released by unit mass of TCE transformed.

Toxicity study

To assess the direct effect of TCE concentration, aerobic granules grown with sodium acetate as the sole carbon and energy source (600 μm mean diameter) were incubated with various concentrations of TCE for 24 h. The granules were washed and resuspended, and the subsequent acetate or phenol-dependent SOUR were measured in duplicates.

Phenol grown aerobic granules were subjected to the same procedure, and the TCE degradation was monitored. The subsequent endogenous respiration rates and phenol-dependent specific oxygen utilization rates (SOUR) were measured as an indication of TCE transformation product toxicity (TPT). The test was done with triplicated samples.

Analytical methods

The measurements of phenol and TCE were as described in Chapter Three.

The specific oxygen utilization rate was obtained by incubating known amount of biomass with phenol or acetate and measuring the dissolved oxygen concentration at suitable time intervals. The oxygen utilization rate was divided by VSS to give SOUR. The endogenous respiration rates were measured in the same way, but without substrate addition, and was deducted from the substrate dependent SOUR. A respirometer (MicroOxymax, Columbus instruments) was also used to investigate the oxygen utilization of the biomass under different treatments.

Chloride ion

The measurement of trace amount of chloride ions was in accordance to the method described by Bergmann et al. (1957). After centrifuge, 15 mL of supernatant from a reaction vial of TCE degradation was combined with 2.5 mL 0.25M $\text{NH}_4\text{Fe}(\text{SO}_4)_2$ in 9N HNO_3 and 2.5 mL ethanol saturated with $\text{Hg}(\text{SCN})_2$. The reaction was allowed to proceed for 10 min, after which the absorbance at 460 nm was measured. Standard curve was obtained by NaCl standard solutions diluted from 1000 mg L^{-1} Cl^- stock solution. The detection limit was 0.1 mg L^{-1} and linear range was measured up to 50 mg L^{-1} with the correlation coefficient of 0.9994. Deionized water was used as the blank.

Formate

Sodium formate was quantified using HPLC (Perkin Elmer) and a column of polypore H (Brownlee columns). The mobile phase used was 0.005 N H₂SO₄ with a flow rate of 0.15 mL min⁻¹. The linear range was measure up to 20 mM with a correlation coefficient (R²) of 0.9992.

4.3 Results

4.3.1 Kinetics of phenol and TCE degradation

The phenol and TCE degradation kinetics were determined by subjecting the granules to various initial phenol or TCE concentrations. The initial removal rates of the substrates were measured and the results are shown below in Figures. 4.1 and 4.2. The kinetic parameters obtained from the modeling using Haldane's equation for phenol, and Michaelis-Menton kinetics for TCE degradation are $V_{\max}=1.59$ mg phenol g SS⁻¹ min⁻¹, $K_S=37.2$ mg L⁻¹, $K_I=461.8$ mg L⁻¹ for phenol; and $V_{\max}=0.0142$ mg TCE g SS⁻¹ min⁻¹, $K_S=1.02$ mg L⁻¹ for TCE.

Therefore, in the absence of substrate inhibition, 1 gram of stable phenol-grown aerobic granules can degrade approximately 1.6 mg phenol in 1 min at the fastest rate. However, the real kinetics incorporating inhibitory effect result in the actual maximum rate V^* of 1.02 mg phenol g SS⁻¹ min⁻¹ at phenol concentration $S^*=131.0$ mg L⁻¹.

Fig. 4.1. Phenol degradation kinetics by phenol-grown aerobic granules. The result was modeled using non-linear regression according to the Haldane kinetics, $V=V_{\max}S/(S+K_S+S^2/K_I)$. (■) the experimental data, representing the average of 3 replicates $\pm$ standard deviation; (—) the fitted curve.

Fig. 4.2. Trichloroethylene degradation kinetics by phenol grown aerobic granules. The data were modeled using Michaelis-Menten kinetics, $V=V_{\max} S/(K_S+S)$. (■) the experimental data, representing the average of 3 replicates $\pm$ standard deviation; (—) the fitted curve.

The Michaelis-Menton kinetics was found sufficient to describe TCE degradation. The maximum rate of TCE transformation by phenol-grown aerobic granules is 0.014 mg TCE per min per gram of biomass, and the half saturation constant is approximately 1 mg L⁻¹ TCE in the liquid phase.

4.3.2 The transformation capacity and the mineralization potential

The concentrations of Cl⁻ ions in the reaction medium after TCE degradation were measured and the results are shown in Fig. 4.3.

Fig. 4.3. TCE mineralization by phenol-grown aerobic granules. A linear correlation was obtained, indicating a release of 2.5 mmol of Cl⁻ per mmol TCE degraded, with an R² of 0.975. (■) the experimental data, representing the average of 3 replicates $\pm$ standard deviation; (—) the fitted curve.

A numeric average of 2.8 mmol of Cl⁻ was calculated to be released by each mmol of TCE degraded, while the slope of the linear regression shown in Fig. 4.3 is 2.5.

According to either value, it could be inferred that almost total mineralization and detoxification of TCE occurred. As the toxicants are most likely electrophilic acyl chlorides (van Hylckama Vlieg et al., 1997), the high degree of dechlorination might indicate less serious toxicity.

The transformation capacity was determined to be $0.011 \text{ g TCE g SS}^{-1}$, that is each gram of SS could transform approximately 11 mg of TCE before the reaction stopped.

4.3.3 Effect of exogenous electron source addition on TCE transformation

Sodium formate or sodium citrate was added to TCE degradation test vials to observe the effect of exogenous electron sources on the instant TCE transformation. The test procedure was similar to the TCE kinetics and toxicity studies, except that a portion of the mineral medium was replaced by concentrated formate or citrate solution to create a desired final concentration. Figures 4.4 to 4.7 show the degradation profiles of TCE with different electron sources.

As shown in Fig. 4.4, the addition of three concentrations of sodium formate, 5, 10, 20 mM didn't have significant effect on the instant TCE degradation (6 mg L^{-1} initial TCE concentration in the liquid), compared to the blank with no formate addition (formate conc. 0 mM). Therefore, in the subsequent tests, the medium formate concentration, 10 mM was used.

Two other TCE concentrations, low (3 mg L^{-1}) and high (15 mg L^{-1}) were further tested with 10 mM formate, and the results are shown in Figures 4.5 and 4.6. Under the initial TCE concentrations of 3 and 15 mg L^{-1} , the addition of 10 mM formate didn't exhibit any significant stimulating effect.

As formate addition didn't appear to enhance the instant TCE degradation under all initial TCE concentrations, another exogenous electron source, sodium citrate was tested. Figure 4.7 shows the result, that it had no significant effect either.

Fig. 4.4. TCE degradation (medium initial concentration) with the addition of 4 concentrations of sodium formate. Initial TCE concentration in the liquid: 6 mg L^{-1} , SS: 2.32 g L^{-1} , formate addition (■) 0 mM, (▲) 5 mM, (●) 10 mM, (×) 20 mM. The data were the average of duplicate samples.

Fig. 4.5. TCE degradation (low initial concentration) with the addition of 10 mM sodium formate. Initial TCE concentration in the liquid: 3 mg L^{-1} , SS 2.3 g L^{-1} ; (■) without formate (▲) with formate. The data were the average of duplicate samples.

Fig. 4.6. TCE degradation (high initial concentration) with the addition of 10 mM sodium formate. Initial TCE concentration in the liquid: 15 mg L⁻¹, SS 1.98 g L⁻¹; (■) without formate (▲) with formate. The data were the average of duplicate samples.

Fig. 4.7. TCE degradation with the addition of 1 mM sodium citrate. Initial TCE concentration in the liquid: 4.4 mg L⁻¹, SS 4.12 g L⁻¹; (■) without citrate (▲) with citrate. The data were the average of duplicate samples.

The data of TCE T_r and T_c change under various exogenous electron sources additions are shown in Table. 4.1. Although the instant TCE transformation rate (T_r) was not affected by either formate or citrate addition, the repeated formate supply during long term TCE degradation appeared to enhance the ultimate TCE transformation capacity (T_c) slightly.

Table 4.1. Effect of exogenous electron sources addition on TCE transformation rate and transformation capacity by phenol-grown aerobic granules (data shown are the average of duplicated samples unless otherwise stated)

exogenous electron source addition	none	Formate			citrate
		5 mM	10 mM	20 mM	1 mM
degradation rate (mg TCE g SS ⁻¹ h ⁻¹) ^a	0.70	0.73	0.74	0.71	0.71
transformation capacity (mg TCE g SS ⁻¹)	0.0115±0.001 ^b	N.D. ^c	0.0145±0.003 ^b	N.D.	N.D.

^a initial TCE concentration in the liquid approximately 5 mg L⁻¹

^b average of triplicated samples ±standard deviation

^cNot determined

4.3.4 Effect of biomass size on TCE and phenol degradation

The granular sludge harvested from the reactors was sieved to different size fractions, and each fraction was tested for their TCE, phenol or formate degradation activities.

The effect of the size of granules on TCE and phenol degradation is summarized in Table 4.2.

Table 4.2. Effect of biomass sizes on TCE and phenol degradation by aerobic granules (data represent the average of duplicated samples)

Size range (μm)	<150	150~300	300~500	500~700	>700
TCE degradation rate (mg TCE g SS ⁻¹ h ⁻¹) ^a	0.88	1.04	0.86	0.82	0.70
TCE transformation capacity (mg TCE mg SS ⁻¹)	0.061	0.039	0.026	0.020	0.018
endogenous respiration rate (mg O ₂ g VSS ⁻¹ h ⁻¹)	12.2	5.6	3.8	2.4	2.6
Formate ^b SOUR (mg O ₂ g VSS ⁻¹ h ⁻¹)	31.1	44.2	37.7	26.7	N.D. ^e
Phenol ^c SOUR (mg O ₂ g VSS ⁻¹ h ⁻¹)	153.7	111.6	66.0	54.0	N.D.
T _C increase with formate addition ^d (%)	21	35	21	30	N.D.

^ainitial TCE concentration approximately 3 mg L⁻¹ in the liquid^binitial formate concentration 10 mM^cinitial phenol concentration 250 mg L⁻¹^dnormalized to T_C determined from samples receiving no formate addition^enot determined

A general decreasing trend was observed with increasing granule sizes for TCE T_r, T_C, endogenous respiration rate and specific oxygen utilization rate (SOUR) on phenol. Granules with the diameter greater than 700 μm could transform 0.018 mg TCE per gram of biomass, while flocs (diameter <150 μm) had a T_C three times higher. The phenol dependent SOUR was also greatly reduced with the increasing size. The formate dependent SOUR and the T_C reliance on formate addition show no significant difference.

4.3.4 Direct effect of TCE concentration on biomass activities

As TCE degradation exerts product toxicity to microbes, the direct toxicity of TCE itself must be studied without the occurrence of TCE degradation. TCE degradation is catalyzed by phenol hydroxylase in this study, and the expression of phenol hydroxylase is inducible. Therefore, if the biomass has never been exposed to phenol, phenol hydroxylase is not induced, and the biomass can degrade neither phenol nor TCE.

Aerobic granules grown on sodium acetate as the sole carbon and energy source don't have the ability to degrade phenol or transform TCE. Acetate grown aerobic granules were exposed to different TCE concentrations from 17 up to 70 mg L⁻¹ in the liquid, to study the direct effect of TCE concentration on granular biomass. No TCE degradation was observed in 24 h of exposure. The medium was purged with air, and the granules were washed with fresh medium and resuspended. The subsequent endogenous respiration rate was measured, and the granules were incubated with acetate or phenol, where the substrate-dependent SOUR were also measured. The results are listed in Table 4.3. No obvious trend of change was observed for the three parameters.

Table 4.3. Effect of TCE exposure on the subsequent endogenous respiration rate, acetate SOUR and phenol SOUR of acetate-grown aerobic granules (duplicated samples)

TCE concentration exposed (mg L ⁻¹ in the liquid)	0	17	35	50	70 ^a
endogenous respiration rate (mg O ₂ h ⁻¹ g VSS ⁻¹)	15.8	13.4	14.2	13.2	13.4
acetate SOUR (mg O ₂ h ⁻¹ g VSS ⁻¹) ^b	44.0	52.5	45.6	43.8	41.2
phenol SOUR (mg O ₂ h ⁻¹ g VSS ⁻¹) ^c	19.6	20.7	29.5	16.6	18.7

^aapproximate value

^bsodium acetate concentration 10 mM

^cdetermined under initial phenol concentration of 250 mg L⁻¹

4.3.5 TCE transformation product toxicity (TPT)

As the exposure to 70 mg L^{-1} of TCE in liquid had no significant effect on the biomass activities, the change of biomass activities after TCE transformation can be solely attributed to the transformation product toxicity (TPT).

The phenol-grown aerobic granules were then incubated with various amounts of TCE and allowed a 24h degradation period. The amounts of TCE transformed were determined and the subsequent endogenous respiration rates and phenol SOURs were measured. Figures 4.8 and 4.9 show the effect of TCE TPT on the two parameters.

Fig. 4.8. The transformation product toxicity effect of TCE on phenol-grown aerobic granules. The endogenous respiration rate and the phenol dependent SOUR (250 mg L^{-1} phenol) were measured in triplicates for granules that had transformed different amount of TCE. (■) endogenous respiration rate; (▲) phenol dependent SOUR.

Fig. 4.9. The transformation product toxicity effect of TCE on phenol-grown aerobic granules (smaller scale, triplicated samples). (■) endogenous respiration rate; (▲) phenol dependent SOUR.

The endogenous respiration rates were found to decrease with the increasing TCE transformed. A drop from 14.3 to 5.8 mg O₂ g VSS⁻¹ h⁻¹ was observed when the biomass transformed 2.73 mg TCE g SS⁻¹, compared to the blank receiving zero TCE dosage. Between 2.73 and 9.82 mg TCE g SS⁻¹ transformed, the respiration rate didn't exhibit significant change (between 4 and 4.8 mg O₂ g VSS⁻¹ h⁻¹). However, when the T_C of biomass was almost exhausted (10.9 mg TCE g SS⁻¹), it further decreased to 3.0 mg O₂ g VSS⁻¹ h⁻¹.

When the biomass transformed 2.73 to 10.9 mg TCE g SS⁻¹, an almost linear decrease of phenol SOUR was observed (from 61.4 to 19.0 mg O₂ g VSS⁻¹ h⁻¹), but a smaller dose of TCE (lower than 2.73 mg TCE g SS⁻¹) increased the phenol SOUR from 46.8 to 61.4 mg O₂ g VSS⁻¹ h⁻¹. In order to confirm this phenomenon, the test was again conducted in a smaller scale (0 to 4.6 mg TCE g SS⁻¹). The results in Fig. 4.9 show similar trends, that the endogenous respiration rate dropped and phenol SOUR rose for the granules transforming 0 to 2.74 mg TCE g SS⁻¹. 1.5 mg TCE g SS⁻¹ seemed to be a threshold beyond which significant changes for both parameters could be observed.

4.3.6 Other studies on TCE TPT

The previous tests on TCE TPT in this chapter used 250 mg L⁻¹ phenol for phenol-dependent SOUR determination, as this concentration was the initial phenol concentration in the reactors and was used throughout the entire study. To evaluate the possible effect of TCE transformation on the response of aerobic granules to different phenol concentrations, two other phenol concentrations were applied to granules after TCE degradation. The phenol-dependent SOURs (mg O₂ g VSS⁻¹ h⁻¹) under various phenol concentrations are shown in Table 4.4.

Table 4.4. Subsequent phenol-dependent SOUR at different phenol concentrations after TCE transformation (average of duplicated samples, unit mg O₂ g VSS⁻¹ h⁻¹)

<div style="text-align: center;"> <i>TCE transformed</i> <i>(mg TCE</i> <i>phenol g SS⁻¹)</i> <i>concentration</i> <i>(mg L⁻¹)</i> </div>			
	0	4.1	8.2
50	63.0	48.5	37.9
250	41.0	34.3	28.5
500	30.3	30.3	22.8

The blank that had received no TCE addition had the highest phenol dependent SOUR under all the three phenol concentrations, especially at the lowest of 50 mg L⁻¹. With the increasing of phenol concentration, the SOURs exhibited a decreasing trend. For the blank, the SOUR with 500 mg L⁻¹ was only half of that under 50 mg L⁻¹. However, for the granules that had transformed TCE, although the general phenol-SOURs were lower than those of the blank, the reduction with higher phenol concentration was less severe.

Finally, to examine if the enhanced phenol uptake after low amount TCE degradation was sustainable, multi dosages of phenol were applied after the TCE degradation. The phenol-dependent SOURs of three consecutive phenol additions to the granules (250 mg L^{-1} each) are listed in Table 4.5. TCE transformation did increase phenol SOUR and this effect was sustainable throughout the 72h of test period, although the SOUR for each sample decreased with longer time of starvation.

Table 4.5. Effect of TCE degradation on the subsequent repetitive phenol degradation activities of phenol-grown aerobic granules (the samples were not duplicated)

TCE degraded (mg TCE g SS ⁻¹)	0	2.72	4.50	5.40	6.03	6.79
average endogenous respiration rate (mg O ₂ g VSS ⁻¹ h ⁻¹)	13.2	13.0	8.4	9.8	8.8	8.9
first phenol SOUR (mg O ₂ g VSS ⁻¹ h ⁻¹) ^a	22.8	46.8	49.5	50.2	46.2	46.6
second phenol SOUR (mg O ₂ g VSS ⁻¹ h ⁻¹) ^b	30.2	44.5	37.3	46.4	34.6	37.5
third phenol SOUR (mg O ₂ g VSS ⁻¹ h ⁻¹) ^c	14.2	28.7	32.6	40.6	32.3	25.6

^aphenol concentration 250 mg L^{-1}

^b24 h after first addition

^c48 h after first addition

4.4 Discussion

4.4.1 Comparison of TCE and phenol degradation kinetics

Kinetic parameters are necessary in assessing the degradation activities of the biomass and in designing reactors, therefore phenol and TCE transformation kinetic behaviors of the granules were first investigated. The results were compared with those of other works as shown in Tables 4.6 and 4.7.

Table 4.6. Comparison of phenol degradation kinetics

$V_{\max}$ (g phenol g SS ⁻¹ h ⁻¹)	K_S (mg L ⁻¹)	K_I (mg L ⁻¹)	microorganisms	Reference
0.06~0.7	0.3~245	3~934.5	pure or mixed cultures	Ong & Bowers, 1990
0.05~1.5 ^a	0.009~0.3	11.3~692	pure cultures	Watanabe et al., 1996b
0.83	1.5	188~315	<i>Acinetobacter</i> species	Hao et al., 2002
0.23	481	212	mixed culture aerobic granule	Jiang et al., 2002
0.10	37.2	461.8	mixed culture aerobic granule	this study

^a estimated according to $1 \text{ mol O}_2 \text{ g dry cells}^{-1} \text{ min}^{-1} = 0.5 \text{ mol phenol g dry cells}^{-1} \text{ min}^{-1}$

The phenol degradation kinetics were modeled using Haldane's equation, $V = V_{\max}S/(S + K_S + S^2/K_I)$, with an R^2 of 0.925. The three parameters $V_{\max}$, K_S and K_I derived from this study were within the ranges of reported values. The maximum specific degradation rate ($V_{\max}$) and the half velocity constant (K_S) were at the lower end of the range, indicating that phenol-grown aerobic granules in this study exhibit comparatively lower but reasonable degradation activity and relatively high affinity towards phenol. The inhibition constant K_I was at the high end (460 mg L⁻¹ as compared to 11 mg L⁻¹ of some pure culture). As mentioned by Jiang et al. (2002), aerobic granules are aggregated cells and can better tolerate the toxicity of high phenol concentration. They are able to retain considerable phenol degradation activity even when the substrate already exerts inhibitory effect.

Michaelis-Menton kinetics was used in simulating TCE degradation, as most previous studies reported that low TCE concentrations (below 30 mg L⁻¹) didn't have inhibitory effect on biomass (Chang & Alvarez-Cohen, 1995ab; Ayoubi & Harker 1998; Park et al., 2002). The TCE degradation kinetics could be sufficiently described by Michaelis-Menton equation up to TCE concentration of 16 mg L⁻¹ in the liquid, with an R^2 of 0.935. Higher concentrations were not used, as they are less likely to be encountered in the environment. The results listed in Table 4.5 show that phenol grown aerobic granules had lower transformation rate and high affinity for TCE. The transformation capacity of TCE was also low but comparable to reported values for phenol or toluene degraders listed in Table 4.7.

Table 4.7. Comparison of TCE co-metabolic degradation kinetics by phenol and toluene degraders

$V_{\max}$ ((mg TCE mg dry cells ⁻¹ d ⁻¹))	K_s (mg L ⁻¹)	T_c (mg TCE mg SS ⁻¹)	microorganisms	reference
0.75	0.39	N.D.	<i>Burkholderia cepacia</i> G4	Folsom et al, 1990
1.0 ^{ab}	N.D.	0.007 ^b	<i>Pseudomonas putida</i>	Heald & Jenkins, 1994
0.2	2.04	0.035	mixed culture	Chang & Alvarez- Cohen, 1995b
0.1	0.35	N.D.	mixed culture	Shih et al., 1996
0.8~1.9 ^b	0.5~3.8	N.D.	pseudomonads pure cultures	Sun & Wood, 1996
0.06~1.3	1.4~105	N.D.	pure cultures	Futamata et al., 2001a
1.4 ^a	N.D.	0.1	<i>Burkholderia cepacia</i> G4	Yeager et al., 2001b
0.02	1.02	0.011	mixed culture aerobic granule	this study

^a initial rate instead of the maximum rate ($V_{\max}$)^b calculated according to 1 g of dry cells containing 0.5 g of protein

4.4.2 Cause for the relatively low TCE T_r and T_C

As mentioned above, aerobic granules have considerable lower $V_{\max}$ for phenol and TCE and T_C for TCE. There could be several reasons for these phenomena. The mechanism of TCE co-metabolism is the catalysis by non-specific enzymes. When these enzymes are monooxygenases (e.g. phenol hydroxylase in this study), the catalysis reaction is shown below in Fig. 4.10 (Fox et al., 1990):

Fig. 4.10. Simplified degradation mechanism of TCE co-metabolism by monooxygenases (Fox et al., 1990).

In the presence of cosubstrates NAD(P)H (reduced nicotinamide adenine dinucleotide) and O_2 , the monooxygenases catalyze the first step in the degradation of TCE to form an intermediate, which subsequently transforms to TCE epoxide. The rate limiting step is believed to be the enzyme reaction (Semprini 1997), as TCE epoxide is unstable and breaks down spontaneously in aqueous solutions (Vogel et al., 1987). Therefore a slow degradation of TCE could be due to 1) insufficient supply of TCE; 2) reducing energy (NADH); 3) oxygen to the enzyme; or 4) an intrinsic slow transformation of TCE by the enzyme.

In this study, the solution of TCE from the gas phase to the liquid phase, or the TCE diffusion in the liquid phase was not likely to limit the rate of the degradation, as vigorous shaking (250 rpm) was provided to the reaction vials, while it was reported that

200 rpm was high enough to ensure TCE mass transfer (Folsom et al., 1990). Similarly, oxygen supply should not be rate limiting either, as a headspace of 40 mL was provided to every 20 mL of reaction medium. Therefore, factors causing low $V_{\max}$ can be the possible insufficient NADH supply, low enzyme activity or the diffusion of TCE from the bulk liquid to the cells.

In order to determine the possible cause(s), the effects of exogenous electron donor addition and the biomass sizes on TCE degradation were studied.

4.4.2.1 Effect of exogenous NADH supply

Formate and citrate are typical NADH producing substrates (Simon et al., 1985). 5, 10 or 20 mM sodium formate, or 1 mM sodium citrate was provided in the reaction medium containing granules, and the instant TCE transformation rates were monitored under various initial TCE concentrations. The results under 5 mg L⁻¹ initial TCE concentration were summarized in Table 4.1. Addition of the exogenous NADH sources didn't have significant effect in enhancing instant TCE degradation, as was evident by the constant degradation rates (around 0.70 mg TCE g SS⁻¹ h⁻¹) in all the reaction vials, with or without exogenous NADH source addition. This indicates that the instant rate of TCE transformation is not limited by electron supply, and compared to the exogenous electron sources, granules preferred to use the endogenous NADH supply (e.g. PHB, as suggested by Henrysson & McCarty, 1993) for short term TCE degradation. Biomass cultivated under SBR mode (such as aerobic granules) is able to accumulate energy storage substances (Beun, 2001). It is likely that aerobic granules have such substances to sustain themselves during famine periods of the operation.

As no limiting effect was observed with different formate concentrations, 10 mM formate was further used to assess its effect on the ultimate transformation capacity. Repetitive additions of 10 mM formate to the reaction vials were found to increase the T_C from 0.0115 to 0.0145 g TCE g SS⁻¹ (26% of increase). This might suggest that as the test prolonged, endogenous NADH supply was exhausted and the biomass relied more on

external reducing energy sources. Residue formate concentrations were measured at the end of the test, and were found to be higher than zero, therefore the exogenous electron donor was not limiting during the test duration. Citrate was not used because it is a 6-C compound, and may support instant biomass growth. New grown biomass could adsorb TCE and distort the test result.

4.4.2.2 Effect of size

As the addition of exogenous electron sources had limited effect in enhancing TCE degradation, the TCE mass transfer from the bulk liquid to the biomass was further investigated as the cause of the low TCE $V_{\max}$ and T_C . The granular biomass harvested from the AGSB reactor was sieved to fractions of different mean sizes, <150, 150~300, 300~500, 500~700, and >700 μm . The morphologies of the biomass fractions are shown in Fig. 4.11.

Fig. 4.11. Morphologies of biomass that was sieved to fractions of different mean sizes.

The fractions were tested for their respective TCE transformation rates and capacities, endogenous respiration rates, formate and phenol dependent SOURs, and their dependence on exogenous electron donor.

As shown in Table 4.2, granular biomass size had significant effect on the TCE T_C , endogenous respiration rate and phenol SOUR. All the three parameters decreased significantly with increasing biomass mean sizes, and larger granules of $>700\ \mu\text{m}$ diameter had only one third of the activities of small flocs ($<150\ \mu\text{m}$). It indicates that the diffusion limitation in bigger granules may cause insufficient TCE mass transfer from the bulk liquid to the inner layers of cells, resulting in an apparent lower specific phenol degradation rate or TCE transformation capacity.

The instant TCE degradation rate and the formate dependent SOUR also decreased with increasing biomass sizes, but to a much lower degree. The $150\sim300\ \mu\text{m}$ fraction, instead of the smallest size ($<150\ \mu\text{m}$) exhibited the highest values for these two parameters. The reason for this phenomenon is not clear. Upon the addition of 10 mM formate, the biomass of all sizes exhibited similar percentages of T_C increase (between 20 to 35%). This might indicate that the biomass from the AGSB reactor had similar storages of endogenous electron supply, regardless of their sizes.

As shown above, the relatively low V_{max} for phenol and TCE, and the transformation capacity for TCE are more likely due to the diffusion limitation in big and compact granules, than to the shortage of electron supply. The activities of small flocs from the AGSB reactor were comparable, or even higher than the previously reported values listed in Table 4.4 and 4.5, therefore ruled out the possibility of low enzyme activities.

4.4.3 TCE toxicity

TCE can exert two kinds of effects on biomass. One is the direct effect of TCE itself, and the other is the transformation product toxicity (TPT).

TCE aerobic co-metabolism was found to produce highly reactive intermediates, which could bind to the non-specific enzymes and other cellular materials, causing the toxic effect (Rasche et al., 1991). Cells that have transformed TCE were found to have lower cell viability (Heald & Jenkins, 1994), general respiratory activities (Chu & Alvarez-Cohen, 1999) and growth substrate degradation rates (Hyman et al., 1995), and the degrees of decreases were positively correlated with the amounts of TCE transformed, not with TCE itself (Alvarez-Cohen & McCarty, 1991a). When the cells had transformed TCE equal to their transformation capacity, *Methylosinus trichosporium* OB3b were found to lose its activity completely, and recovery was not possible even after 1 week of incubation with its growth substrate, methane (Chu and Alvarez-Cohen, 1999). The toxic effect is a serious problem in the application of aerobic co-metabolism in TCE degradation, therefore the response of aerobic granules to TCE TPT is of special interest.

Before studying the toxicity of TCE transformation, the effect of the concentration of TCE itself was investigated. Two possible effects could be exerted by TCE itself on biomass in contact with TCE, the solvent stress and the induction effect. Organic solvents with a log K_{OW} between 1.5 and 3 may generally cause cytoplasmic membrane-associated toxicity (Sikkema et al., 1995). TCE has a log K_{OW} of 2.61, and could have a concentration-dependent damage on cells when present in liquid phase. TCE was also found to induce the expression of toluene oxygenases in various pure cultures (Heald and Jenkins, 1994; McClay et al., 1995; Leahy et al., 1996; Shingleton et al., 1998 and Park et al., 2002). In other words, TCE could induce its own degradation, as well as the degradation of the primary substrate, e.g. toluene.

To determine if the TCE concentrations used in the tests had any toxic or inducing effect, aerobic granules grown with sodium acetate as the sole carbon and energy source were

obtained and exposed to different concentrations of TCE for 24 h, and the subsequent endogenous respiration rate, acetate dependent SOUR and phenol SOUR were measured.

The results listed in Table 4.3 showed no significant changes in the three parameters. As the endogenous respiration rate and the acetate SOUR were not affected by TCE with the concentration of up to 70 mg L^{-1} in the liquid, the solvent stress was not likely to harm the aerobic granules. Similarly, if TCE had any inducing effect on the acetate-grown granules, those exposed to higher concentration of TCE should have a higher phenol SOUR afterwards. Therefore, the effects of TCE concentration itself could be considered as negligible, at least within the concentration range used in this study.

The TCE transformation product toxicity was subsequent investigated. Various amounts of TCE were injected to reaction vials containing phenol-grown aerobic granules (the highest concentration in liquid was 30 mg L^{-1}), and the degradation was carried out for 24 h. The subsequent endogenous respiration rates and phenol SOUR of the washed granules were shown in Figures 4.8 and 4.9. It could be seen from Fig. 4.8 that both parameters decreased with increasing TCE transformed. The endogenous respiration rate exhibited a sharp drop and then a steady decline throughout the test range ($0\sim 10.9 \text{ mg TCE g SS}^{-1}$, equal to $0\sim 95\%$ of T_C). The phenol SOUR first rose by around 50% then decreased in a linear fashion. A smaller test range ($0\sim 40\%$ of T_C) was used in obtaining Fig 4.9, where a similar trend showed an increase of phenol SOUR and a drop of the endogenous respiration rate. The threshold seemed to be around $1.5 \text{ mg TCE g SS}^{-1}$ (13% of T_C), beyond which the endogenous respiration rate was affected by TCE degradation, and the growth substrate intake was enhanced.

Compared to the results of previous studies on TCE transformation product toxicity, the phenol grown aerobic granules showed a unique response to TCE TPT, which could be explained as follows.

First, most of the previous studies (Alvarez-Cohen & McCarty, 1991b; Chu & Alvarez-Cohen, 1999) reported that when the transformation capacity of certain biomass was

almost spent (ratio of TCE transformed to biomass exceeded 95% of T_C), the growth substrate degradation activity and respiratory activity dropped to nearly zero. In this study, the transformation capacity of phenol-grown aerobic granules without the addition of formate was 11 mg g SS^{-1} . When the granules transformed $10.9 \text{ mg TCE g SS}^{-1}$ (the last data point in Fig. 4.8), neither the endogenous respiration rate nor the phenol-dependent SOUR was reduced to zero. Granules still retained one third of their original endogenous respiration activity and half of its phenol degradation activity.

Second, TCE transformation inactivates cells by generating electrophilic acyl chlorides that can bind to cellular materials. The bound cellular components are covalently modified, their structures are changed, and they are considered no longer functional. The binding and modification are believed to be irreversible. Recovery of biomass from TCE TPT is possible in some studies, by incubating the biomass with its growth substrate or other energy yielding substrate. Cells use the energy and carbon derived from the degradation of growth substrates to resynthesize components to replace the inactivated cellular materials. As *de novo* synthesis of cellular materials was said to be essential in the recovery of biomass from TCE TPT (Yeager et al., 2001a), a lag phase should be observed when the biomass degrades its growth substrates after it transforms TCE. During this lag phase, cells recover by resynthesis of new proteins within preexisting cells rather than by an uncharacteristically rapid division of a subset of noninactivated cells (Hyman et al., 1995). The higher the inactivation degree is, the longer the lag phase should be. However in this study, phenol degradation began immediately after 24 h of TCE degradation and starvation from phenol, even when the T_C was almost exhausted. No lag phase was observed, indicating that the resynthesis of new proteins probably didn't occur.

Third, the results in this study indicated an extraordinary trend in the phenol SOUR change. The phenol dependent SOUR generally decreased with the increasing amount of TCE transformed, but the granules exhibited an enhanced phenol uptake when they degraded between 1.37 to $3.65 \text{ mg TCE g SS}^{-1}$ (12~32% of T_C). Only when the TCE transformed to biomass ratio was higher than $6.55 \text{ mg TCE g SS}^{-1}$ did the phenol SOUR

dropped below the respective value of the blank (no TCE addition, the first data point in Fig. 4.4). However, Chu & Alvarez-Cohen (1999) reported that when pure culture *Methylosinus trichosporium* OB3b degraded TCE, TCE oxidation caused sMMO-catalysed activity and respiratory activities to decrease linearly with the amount of substrate degraded. The work of Yeager et al. (2001b) indicated that TCE degradation resulted in a precipitous loss of cell culturability, and the loss was in exponential relationship with the amount of TCE transformed.

Therefore, the aerobic granules exhibited an unusual response to TCE TPT. Small amounts of TCE transformation enhanced the subsequent phenol degrading rates; only higher degrees of TCE TPT could decrease the granules' phenol uptake activities; and recovery was possible even when the transformation capacity was spent. This is the first time it is reported that TCE degradation could have stimulating effect on biomass's performance in the subsequent growth substrate degradation.

4.4.4 Possible causes of the unique response to TCE TPT

The possible reason for the aerobic granules' unique response to TCE TPT was further explored in this study. A previous study also reported usual TCE TPT effect on phenol degraders. Morono et al. (2004) observed that *Pseudomonas putida* F1 exhibited an immediate toluene degradation activity after it stopped transforming TCE, and proposed a "pseudo-inactivation" theory. It was suggested that the enzyme transforming TCE might be inactivated by the binding of TCE and its products, and the subsequent primary substrates addition can "flush out" these toxicants, restoring the enzyme activity. Yet this theory cannot explain the enhanced phenol uptake by granules transforming small amounts of TCE observed in this study. To tentatively explore the cause of this phenomenon, previous attempts to model the transformation product toxicity were examined.

Alvarez-Cohen and McCarty (1991c) developed a TCE co-metabolism model where the transformation capacity (T_C) was used to describe the TCE product toxicity. This model

implied a linear relationship between the amount of TCE transformed and the quantity of biomass inactivated. The fraction of cells that have transformed TCE equal to its T_C is considered as totally dead. Others proposed that the toxicity involved the enzyme inactivation, inhibition and recovery rather than the whole cell (Ely et al., 1995).

A general model of TCE turn-over dependent toxicity can be described by the mechanism introduced by Hyman & Arp (1992), although it was originally used for suicide substrate. The mechanism is depicted below in Fig. 4.12.

Fig. 4.12. Simplified mechanism of TCE transformation product toxicity. **Substance:** A-enzyme; B-TCE; C-transformation product (also the reactive species); D-other cellular materials; E-non-toxic end products; F-cellular membrane; G-bulk liquid; H-another cell. **Process:** a-binding of TCE to the non-specific enzyme; b-transformation of TCE to the catalysis product by the enzyme and the release of the product from the active site; c-binding of the product to the enzyme active site and the inactivation of the enzyme; d-binding of the reactive species to other cellular materials and the inactivation; e-spontaneous detoxification of the reactive species to form non-toxic end products; f-diffusion of the reactive species.

After the substrate (e.g. TCE) binds to the enzyme, the catalysis produces an activated reactive species, which can either bind to the enzyme or detach from it. The bound enzymes become covalently modified and inactivated.

The reactive species has three fates: to bind to other cellular materials and inactivate them, to undergo nonbiological transformation and turn into a stable non-reactive product, or to diffuse out of the cellular membrane into the bulk medium. The reactive species in the bulk liquid may again experience a similar detoxification or enter other cells and inactivate them. Therefore the toxicity effect of TCE transformation is a profound phenomenon, which could depend on the TCE transformation rate, the enzyme affinity for TCE and its reactive species, the enzyme structure, degradation pathways and products, and the intra- and inter-cellular environment.

According to this reactive species toxicity model, the robustness of phenol-grown aerobic granules to the TCE turn-over dependent toxicity might have several causes. First, the low transformation rate of TCE may slow down the production rate of the toxicant(s) (the reactive species), giving the cells more potential for recovery. Second, the mixed microbial population in aerobic granules gives rise to diverse enzyme affinities to the toxicants and enzyme structures, and might also produce different products from TCE. Some of the products might not be toxic, and some types of phenol hydroxylases might have higher resistance to the covalent modification by the toxicants. Third, the complex inter- and intra-cellular environment present in the granules may provide opportunities for the spontaneous detoxification of the reactive species after they are produced. Fourth, the compact structure of the aerobic granules may act as a protective barrier against the diffusive toxic products. Fifth, the possible endogenous energy storage might provide the cells with simultaneous recovery as the inactivation occurs.

In order to evaluate the possibilities listed above, a test was carried out in which the phenol degrading granules were mixed with acetate-grown activated sludge flocs. 25 mL Phenol-grown aerobic granules (mean size 670 μm) were mixed with 10 mL sodium acetate grown flocs (mean size 148.5 μm), and the mixtures were incubated with various

amounts of TCE. After TCE was degraded, the mixtures were sieved to separate the granules and flocs. The acetate-dependent oxygen consumption rates of the flocs were measured in duplicates and the result is shown in Fig. 4.13 below. The aim of this test is to study the effect of TCE TPT on the surrounding cells. For this purpose, the surrounding cells must not be harmed by TCE directly, i.e. they didn't degrade TCE or were affected by TCE solvent stress. Acetate grown flocs were chosen in this test, as they meet the requirements stated above, and also because they didn't have the granular structure and diffusion barrier that could possibly protect them from TCE TPT.

Fig. 4.13. Effect of TCE degradation on the surrounding cells that didn't transform TCE. Data represent the average of duplicated samples.

It can be seen from Fig. 4.13 that the TCE transformation by the phenol-grown aerobic granules in the mixture didn't significantly affect the surrounding non-TCE-transforming acetate-grown flocs. Previous studies have shown that when cells not transforming TCE were incubated with TCE and TCE transforming cells, TCE TPT could have negative effect on the non-TCE degrading cells too. A decrease in cell culturability (Yeager et al., 2001b) and fermenting activity of *Saccharomyces cerevisiae* (Reij et al., 1995) were

observed. The reason for such toxicity effect on cells not degrading TCE was indicated to be the diffusive nature of the toxicants, which can leave the TCE degrading cells and enter the bulk liquid.

Therefore it could be inferred that in this study, the toxicants produced from TCE transformation didn't reach the acetate-grown flocs. They were either detoxified within the phenol degrading granules or in the bulk liquid.

The increased phenol-dependent SOUR after small amount of TCE transformation might be explained by the model developed by Ely et al. (1995). It was proposed that enzyme recovery was possible even during TCE degradation, and the more severe the inactivation was, the higher was the "driving force" for cellular recovery. The higher phenol intake ability after TCE transformation may indicate that the cells were trying to degrade more growth substrate and obtain energy for the repairing of inactivated cellular materials. As more TCE was degraded, such a driving force was balanced by the real inactivation effect, and the granules finally exhibited lowed phenol SOUR.

4.4.6 Phenol degradation after TCE transformation

According to the TCE co-metabolism models introduced above, TCE transformation harms the cells that carry out TCE degradation. The worst situation is that cells are totally inactivated and dead. But when less TCE is transformed, sometimes cells can be recovered by incubating them with their growth substrate, or other energy yielding substances (e.g. glucose). It was suggested that cells recover by synthesize new cellular materials (*de novo*) to replace the inactivated ones.

In this study, phenol is used as the growth and energy yielding substrate. One problem arise from it is that what phenol concentration should be used in the cellular recovery. Phenol is a toxic compound, and could have detrimental effect on cells when the concentration is too high. In the work of Yeager (2001b), it was discovered that when TCE TPT-harmed cells were incubated with lactate or phenol for recovery, cells in the former case took less time to recover than the latter. It was postulated that damaged cells

might be more sensitive to phenol toxicity, and the concentration of phenol which was harmless to normal cells might be fatal to the injured cells.

In this study, 250 mg L⁻¹ phenol was used, because it was the initial phenol concentration of the operation cycles in the AGSB reactor. To determine if TCE transformation results in higher sensitivity of the granules to phenol toxicity, another two phenol concentrations were tested (50 and 500 mg L⁻¹).

Table 4.4 lists the phenol-dependent SOURs of TCE-exposed aerobic granules under the three phenol concentrations. For the blank receiving no TCE dose, the SOUR with 50 mg L⁻¹ phenol was 62.0 mg O₂ g VSS⁻¹ h⁻¹, and the SOUR under 250 and 500 mg L⁻¹ was 66% and 49% of this value, respectively. For aerobic granules degrading 4.1 or 8.2 mg TCE g SS⁻¹, the SOURs under all three phenol concentrations were lower than those of the blank. However, the percentage of the SOUR with 250 and 500 mg L⁻¹ to that with 50 mg L⁻¹ phenol were higher (71% and 63% for 4.1 mg TCE g SS⁻¹, 75% and 60% for 8.2 mg TCE g SS⁻¹). Therefore, it can be concluded that TCE transformation didn't increase aerobic granules' sensitivity to phenol toxicity. Granules that have transformed TCE didn't exhibit lowered phenol degradation activity under higher phenol concentrations, compared to granules that have not been exposed to TCE.

As stated above, smaller dosage of TCE could enhance the subsequent phenol dependent SOUR. However it was not clear whether this stimulating effect was a temporary phenomenon or could last longer than one phenol addition. To answer this question, three consecutive phenol additions were applied after the granules had degraded TCE (data see Table 4.5). For all the granule samples that had transformed TCE (2.7~6.8 mg TCE g SS⁻¹), an enhanced phenol-dependent SOUR was observed for all the three phenol dosages (250 mg L⁻¹ each). As the whole test duration was 72 h, the later two phenol SOURs were reduced due to prolonged starvation. However, the enhancement of TCE degradation on subsequent phenol SOUR was observed throughout the test period, which indicates that this effect is sustainable.

4.5 Conclusions

In this chapter, the detail characterization of TCE degradation behavior by stable phenol-grown aerobic granules was introduced. The results obtained from the experiments can be concluded in the following table:

Table 4.8: Summary of the test results in this chapter

Purpose of the test	Prodedure of the test	Results and conclusions
To know the kinetic parameters of phenol degradation	Granules incubated with different concentraions of phenol and specific phenol degradation rates measured	$V_{\max}=1.59 \text{ mg g SS}^{-1} \text{ min}^{-1}$ $K_S=37.2 \text{ mg L}^{-1}$ $K_I=461.8 \text{ mg L}^{-1}$
To know the kinetic parameters of TCE degradation	Granules incubated with different concentraions of TCE and specific TCE degradation rates measured	$V_{\max}=0.0142 \text{ mg g SS}^{-1} \text{ min}^{-1}$ $K_S=1.02 \text{ g L}^{-1}$
To know the TCE transformation capacity of the mature granules	Granules incubated with TCE until no more TCE degradation observed	$T_C=0.011 \text{ g TCE g SS}^{-1}$ without exogenous electron source
To evaluate the TCE mineralization potential by the granules	Granules incubated with TCE and the Cl^- released measured	2.8 mol Cl^- released by 1 mol TCE degraded
To know the effect of exogenous electron source on TCE transformation rate and capacity	Sodium formate and sodium citrate added to granule/TCE mixture and the subsequent TCE degradation monitored	Exogenous electron sources didn't have significant effects on instant TCE T_r , but slightly increase the T_C
To know the effect of granule size on phenol/TCE degradation activities	Granules sieved to different size ranges and their phenol /TCE degradation activities mornitored	Size of the granules negatively affects the TCE/ phenol degradation rates, TCE T_C and endogenous respiration rates
To see TCE exerts solvent stress on granules	Acetate granules incubated with TCE and the subsequent acetate SOUR measured	TCE didn't have significant solvent stress on granules up to the concentration 70 mg L^{-1}

Table 4.8: Summary of the test results in this chapter (continued)

To see if TCE can induce phenol hydroxylase activities in granules	Acetate granules incubated with TCE and the subsequent phenol SOUR measured	TCE didn't have significant induction effect on granules up to the concentration of 70 mg L ⁻¹
To study the TCE transformation toxicity effect on phenol granules	Phenol granules incubated with various amounts of TCE and the subsequent phenol and endogenous SOUR measured and linked with TCE transformation	small amounts of TCE transformation enhanced the phenol uptake ability, higher degree of TCE degradation decrease the subsequent phenol and endogenous SOUR
To see if the enhancing effect of small TCE dosage on subsequent phenol uptake ability is sustainable	Repetitive dosage of phenol applied after TCE degradation and the phenol SOUR monitored	The enhancing effect was sustainable throughout three phenol additions and 72 h test duration
To see if TCE degradation increases granule's sensitivity to phenol toxicity	Different concentrations of phenol applied to granules after TCE degradation and SOUR monitored	TCE degradation didn't increase granule's sensitivity to phenol toxicity
To explore the nature of toxicants produced from TCE transformation	Phenol granules and acetate sludge mixture incubated with TCE, and the acetate sludge measured for acetate SOUR	The toxicants produced by TCE degradation didn't affect the surrounding non-TCE-degrading sludge, probably detoxified within the phenol granules

The phenol-grown aerobic granules had a unique response to the TCE turn-over dependent toxicity, in that small amounts of TCE transformation could enhance the phenol uptake ability of the granules. Such effects were sustainable and their causes were tentatively explored.

When higher dosage of TCE was applied to the granules, the subsequent phenol dependent SOUR was decreased in a linear fashion, but not diminished even when the transformation capacity was spent. TCE degradation didn't reduce the granules' resistance to phenol toxicity.

The phenol-grown aerobic granules' unique response to TCE TPT is a great advantage. The transformation product toxicity has always been a priority concern of researchers, and big problem for reactor design and operation. It reduces the treatment efficiency, increases consumption of primary substrates and complexity of operation, and lowers the sustainability of the reactor. Previous studies all showed that TCE transformation can compromise all kinds of metabolic activities of pure and mixed cultures, even kill cells. Recovery is often time-consuming and expensive, as it involves lengthy incubation of the harmed cells with the primary substrates, therefore lowers the operational efficiency of TCE treatment reactors. It would be much simpler to discharge the TCE-injured cells after transformation, regardless of their state of activity and potential to recover. However it can increase the operational cost significantly, due to the excessive use of primary substrates to grow new batch of cells to degrade TCE.

Phenol grown-aerobic granules exhibited unprecedented resilience to TCE transformation product toxicity. First, the phenol degradation activity was not diminished even when the granules had transformed TCE equal to the T_C , which means it's difficult to "kill" the phenol degraders embedded in the granules. The robustness of aerobic granules, possibly due to its compact structure, makes the recovery possible after TCE transformation. Second, only higher dosage of TCE can reduce the phenol uptake ability of aerobic granules, while lower degree of TCE degradation stimulates phenol-dependent SOUR. This means that recovery of aerobic granules after TCE transformation can be conducted after the granules have transformed relatively large quantity of TCE, and within a large TCE transformed to biomass ratio. Last but not least, degradation of phenol by aerobic granules immediately resumed after TCE degradation and starvation, which could enhance the treatment efficiency, as no time is required for the de novo synthesis of cellular materials to replace the damaged ones.

CHAPTER FIVE

ISOLATION OF PHENOL DEGRADERS FROM PHENOL-GROWN AEROBIC GRANULES AND CHARACTERIZATION OF THEIR PHENOL/TCE DEGRADATION ACTIVITIES

5.1 Introduction

In Chapter Three, the changes of TCE degradation behavior during granulation exhibited by phenol-degrading biomass were studied. In Chapter Four, the activities of stable phenol-grown aerobic granules towards TCE co-metabolism were further explored. Subsequently, it was intended in this chapter to identify various bacteria in the granules, and their respective phenol and TCE degradation activities.

For this purpose, isolation of pure phenol-degrading bacteria was conducted in this chapter with the stable aerobic granules as the source of inoculant. The isolation of phenol-degrading bacteria from phenol-grown aerobic granules had been conducted once by Jiang et al. (2004b), and the ten isolates obtained were designated as the first batch, in contrast with the second batch obtained in this study. The phenol degradation kinetics of the first batch isolates had been studied, but their TCE degradation behaviors were unknown. Therefore, it was intended that in this study, the phenol and TCE degradation kinetics, TCE transformation capacities and other characteristics of the two batches of phenol-degrading pure cultures were to be studied and compared. And the information gathered could be an indication of the functional diversities of bacteria in the granules.

In previous studies, the phenol and TCE degradation kinetics of 22 bacterial pure cultures isolated from contaminated soil were studied (Watanabe et al., 1996b; Futamata et al., 2001a). It was found that according to the phenol kinetic behaviors of the isolated bacteria, especially their K_S and K_I (half-saturation constant and inhibition constant), the pure cultures could be divided into three groups: high K_S , med K_S and low K_S groups. Later it was discovered that the TCE kinetics were in parallel to the phenol kinetics, that is, bacteria having high K_S to phenol also had high K_S to TCE, and vice versa (Futamata et al., 2001a).

Bacteria of low K_S to phenol and TCE were suggested to be preferable in TCE co-metabolic degradation, as they could eliminate TCE to a higher extent and with high rate. However, TCE co-metabolism is a complicated process, the success of which does not depend on the bacterial kinetic behavior alone. Other than the K_S of a pure culture, its V_{max} , transformation capacity and transformation yield (T_y) are also the important parameters in evaluating its performance in TCE co-metabolism.

Therefore the aims of this chapter were, first, to isolate the bacteria pure cultures that were culturable and of numerical abundance within the granules; second, to know how fast they could degrade phenol and TCE, and the kinetics; third, to determine their TCE transformation capacities and growth yields on phenol; and fourth, to select pure cultures that were of higher activities in TCE co-metabolism.

In the kinetic studies, the V_{max} values indicated the specific degradation rates of the isolates, and the K_S indicated their affinities to the substrates. Genrally a bacterial pure culture with high V_{max} and proper K_S is favored for the substrate degradation process, as it can rapidly decrease the concentration of the target compound. A proper K_S means that the pure culture should have the highest degradation rate within certain substrate concentration range, and the range is determined by real application, e.g. continuous or batch process. Generally, a low K_S indicates high affinity.

Pure cultures that could stand TCE transformation product toxicity are preferred, therefore the transformation capacities of the pure cultures were determined and compared. Another parameter of the isolates was also considered, that was the growth yield on phenol. Phenol is the growth substrate for all the TCE transforming bacteria in this study. From an economical aspect, bacteria degrading more TCE while consuming less phenol are better candidates for TCE co-metabolism application. The transformation yield (T_y) is an indication of growth substrate consumption with per unit of TCE transformation.

Therefore, pure cultures were isolated, purified, screened and selected according to their phenol kinetics, TCE kinetics, transformation capacities and growth yields. The objective was to obtain knowledge on the physiological and functional diversity of phenol-degrading bacteria in the aerobic granules, to identify their phenol and TCE degradation activities, and to select candidate for possible TCE co-metabolism application.

5.2 Materials and methods

Chemicals and media

Solid media for plate counting and purification

Four sets of agar plates were used in the isolation process. The first set was for total plate counting, which was prepared by dissolving 23.5 g plate count agar (Difco 247940) in 1 L of water. For the other three sets, 1.2% (12 g in 1 L of water) American bacteriological agar (Pronadisa) was used. It was supplemented with mineral salts, vitamin and micronutrients according to the formula of Jiang et al. (2004b). Phenol was supplied as the carbon and energy source to the second and third sets of agar plates (mineral phosphate buffer-phenol and mineral phosphate buffer-phenol-TCE) at the concentration of 500 mg L⁻¹. The former sets were incubated in the normal air atmosphere, and the latter in a desiccator of 8 L volume. A beaker containing the mixture of 30 mL TCE-saturated water and 20 mL plain water was placed in the desiccator. As TCE is a volatile

compound, the TCE dissolved in the water will evaporate and distribute in the liquid and gaseous phases, the equilibrium of which could be described by the Henry's law constant (refer to Chapter Three). The equilibrated TCE concentration in the desiccator's atmosphere was calculated to be approximately 5 mg L^{-1} in the air. The TCE solution in the beaker was replaced every two to three days. The fourth set, mineral phosphate buffer (MP)-TCE plates were also incubated under the same TCE atmosphere, but were not supplied with phenol in the agar.

Liquid media for growing isolates

MP medium supplied with 250 mg L^{-1} phenol was used to grow isolates in the liquid for further investigation.

Preparation of inoculum and spreading of plates

Granules were harvested from the reactors, mixed and washed twice with sterilized MP medium. They were then pounded to smaller particles with a sterile mortar and pestle for at least 20 min. The grinded sludge was checked with microscopy to ensure that the cells are sufficiently separated, so that one colony on the plate grew from one cell only. The sludge was collected and suspended in fresh MP medium, and the concentration of biomass was measured. The same mortared sludge sample was used to prepare the serial dilutions for inoculation of plates. $100 \mu\text{L}$ of each dilution was placed on one plate and was spread on the agar surface. The colonies appeared on the agar surface were periodically counted. The counting method of the plates was according to Barnett (1990).

Purification and isolation

After the colonies appeared on the serial dilution plates, some of them were picked and streaked on fresh plates for purification. The colonies selected for further investigation were from the phenol-MP and phenol-MP-TCE plates, and the total count agar plates were not used for isolation. The principles for selection were:

- Fast growth, big colony, early appearance on the plates
- Representative morphology
- Growth on the highest dilution plates

According to these criteria, colonies were picked from phenol or phenol-TCE plates and streaked on phenol-MP plates in duplicate. The duplicated plates sourced from phenol-TCE plates were incubated in normal and TCE atmosphere each. The colonies grown on the newly streaked plates were examined by eyes and by microscopic observation after gram staining. Colonies that were of different morphologies on one plate, or were found to contain more than one type of bacteria under microscope were streaked again on fresh plates. When there was only one morphology on one plate, and the colony picked from the plate was uniform under microscopic observation after gram staining, the culture was considered as pure. The differentiation and description of colony morphologies were according to Benson (2002).

Differentiation between isolates of similar morphology

As pure cultures of similar morphologies can actually be genetically different, it was necessary to use culture-independent methods to confirm the results of visual inspection. Repetitive extragenic palindromic sequence-polymerase chain reaction (REP-PCR) was adopted for this purpose (Jiang et al., 2004b). Colonies confirmed to be pure by visual observation were picked from agar surface, and cells were suspended in 100 μ L 0.05M sterile NaOH and incubated at 95°C for 15 min. The alkaline-extracted DNA was used as the template for REP-PCR fingerprinting. The PCR mixture containing buffer, DNA template, dNTPs, BOX AIR primers (5'-CTACggCAAggCgACgCTgACg-3') and Taq-polymerase were subjected to 30 to 35 PCR cycles and the results were visualized on agarose gel after 17 h of electrophoresis. The pure cultures of similar morphologies were placed together and their bands on the gel were compared. Only when two isolates had all the bands at the same positions that they were considered as the same bacteria, as previous studies reported REP-PCR can differentiate between closely related strains of the same species (Dombek et al., 2000).

Growth and preparation of pure culture for kinetic studies

Shake flasks containing MP medium were inoculated with isolates from agar plates, and 250 mg L⁻¹ phenol was supplied as the carbon and energy source. The flasks were placed on orbital shakers and shaken at the speed of 150 rpm. A dilution rate of 0.33 to 0.67 d⁻¹ was adopted to ensure that the bacteria were in healthy growth. Phenol concentrations were measured before the dilution to ascertain the complete degradation of phenol. Cells were collected by centrifugation at 3300 rpm for 15 min, washed with MP medium once, and resuspended in the same medium at various concentrations for kinetic studies. The purity of the isolates was checked at the beginning and the end of incubation by microscope, and no obvious contamination was found.

Phenol and TCE kinetic studies and TCE transformation capacities

Phenol kinetics were expressed as the specific oxygen utilization rates versus phenol concentrations. They were determined by suspending various amount of biomass in 67 mL biological oxygen demand (BOD) bottles, and monitoring the disappearance rates of oxygen with or without phenol of different concentrations.

For the determination of TCE kinetics and transformation capacities of the isolates, see Chapter Four.

Determination of growth yield

The growth yields of the isolates PG01 to PG10 were determined according to the methods described by Jiang et al. (2004b). The specific growth rates were divided by the specific phenol degradation rates to obtain the observed growth yields (Y_{obs}). For the pure cultures isolated in this study, the growth yield was measured by inoculating 25 mL MP medium containing 250 mg L⁻¹ phenol with individual isolates, and incubating the mixtures on orbital shakers (150 rpm). The dry weights (DW) and phenol concentrations were determined at the beginning and end of the incubation, and the true growth yields were calculated as the DW increase divided by phenol consumption. As different methods were used to obtain the growth yields data for the two batches of isolates, the

data were not directly comparable. However, they could still serve as an indication of how much biomass could be produced by unit mass of phenol degraded.

Analytical method

Gram staining

A gram staining kit (BD) was used. The procedure was: crystal violet 1min; rinse with water; iodine 1min; rinse with water; decoloring with isopropanol to acetone 75%:25% (v:v); rinse and safranin 1min; rinse and dry.

Measurement of biomass growth

The dry weight of biomass was determined by filtrating cell suspensions through 0.20 μm pore size membrane filters (cellulose acetate, Waterman), and drying the filters at 80 °C overnight.

Cell surface hydrophobicity

The cell hydrophobicities of the pure cultures were indicated by their contact angles with water. A contact angle system OCA 20 (Dataphysics) was used for the measurement, and the filters containing dried biomass were put in contact with a drop of Milli-Q water. The images were captured by the system software and the angles formed by the water drop and the filter surface were calculated.

5.3 Results

5.3.1 Isolation of pure cultures from phenol-grown aerobic granules

Four sets of agar plates were used in the isolation procedure.

1. Total plate count agar
2. Phenol-MP agar
3. Phenol-MP agar under TCE atmosphere
4. MP agar under TCE atmosphere

The isolation under the first condition gave the total viable count of the culturable microbial population within the aerobic granule. Combining it with the result under the second condition, the percentage of phenol degraders within the total population can be roughly estimated.

The third condition was adopted to observe if the TCE addition in the incubation atmosphere could cause difference in the isolation results, e.g. viable count, representative morphologies and microbial diversity. The fourth condition provided TCE as the sole organic carbon source for the isolation, the aim of which was to explore if any microorganism can grow on TCE as the carbon and energy source.

The plate counts versus incubation time were shown in Figures 5.1 to 5.4.

Fig. 5.1. Increase of plate count with incubation time on the total count agar plates. (■) 10^{-5} dilution (▲) 10^{-6} dilution (●) 10^{-7} dilution (×) 10^{-8} dilution (+) 10^{-9} dilution.

Fig. 5.2. Increase of plate count with incubation time on the phenol-MP agar plates. (■) 10^{-4} dilution (▲) 10^{-5} dilution (●) 10^{-6} dilution (×) 10^{-7} dilution.

Fig. 5.3. Increase of plate count with incubation time on the phenol-MP-TCE agar plates. (■) 10^{-4} dilution (▲) 10^{-5} dilution (●) 10^{-6} dilution (×) 10^{-7} dilution.

Fig. 5.4. Increase of plate count with incubation time on the MP-TCE agar plates. (■) 10⁻² dilution (▲) 10⁻³ dilution (●) 10⁻⁴ dilution (×) 10⁻⁵ dilution (-) 10⁻⁶ dilution.

Although no carbon source other than TCE was provided to the TCE-MP agar plates, many colonies arose in those cultures. As no bacteria have ever been documented to grow on TCE, it was likely that those colonies grew on the trace amount of carbon in the chemicals constituting the mineral medium or in the inoculum (oligotrophic), or on the CO₂ present in the atmosphere (autotrophic). To examine if the colonies really grew on TCE, five colonies of representative morphologies were selected from the 10⁻⁶ serial dilution plates and suspended in liquid MP medium. TCE was provided and the possible growth was monitored for more than 3 months. No obvious turbidity was observed in the test vials, therefore the colonies appeared on the TCE-MP agar plates were more likely oligotrophs or autotrophs.

The plate counting data of the first three sets of agar plates are shown below in Table 5.1.

Table 5.1. Plate counts of total count, phenol-MP and phenol-MP-TCE agar plates

dilution	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
total count agar	N.D. ^a	459	56	12	16	2
	$5.5 (\pm 2.7) \times 10^7$ colonies mg VSS ⁻¹					
Phenol-MP agar	690	218	26	1	N.D.	N.D.
	$2.1 (\pm 1.0) \times 10^7$ colonies mg VSS ⁻¹					
Phenol-MP agar under TCE atmosphere	685	63	2	0	N.D.	N.D.
	$6.2 (\pm 1.1) \times 10^6$ colonies mg VSS ⁻¹					

^a not determined

5.3.2 Purification and characterization of bacteria of numerical abundance

8 types of representative colony morphologies were found on the phenol-MP agar plates, the description of which are shown below in Table 5.2.

Table 5.2. Representative morphologies appearing on phenol-MP agar plates

No.	color	diameter (mm)	configuration	margin	Elevation
1	yellow	3	concentric	undulate	Hilly
2	lemon yellow	1	round	smooth and entire	drop-like
3	lemon yellow	1~2	scalloped	lobate	Flat
4	transparent	2	irregular and spreading	undulate	Flat
5	milky white, semi-transparent	1	round	smooth	drop-like
6	milky yellow, semi-transparent	2	concentric	smooth	raised
7	milky yellow	2~3	round	entire	convex
8	milky brown	3	round with raised margin	entire	raised

The abundance of the representative colonies on the plates of every serial dilution is shown in Table 5.3.

Table 5.3. The numerical abundance of the representative morphologies on serial dilution plates of phenol-MP agar

	10^{-7}	10^{-6}	10^{-5}	10^{-4}
1	33%	30%	1%	0
2	67%	30%	25%	<5%
3	0	0	5%	5~10%
4	0	30%	25%	50~60%
5	0	0	30%	30~40%
6	0	0	10~15%	<1%
7	0	0	0	5~10%
8	0	10%	1%	<1%

Similarly, the data of representative morphologies and their numerical abundance were obtained for the phenol-MP agar plates incubated under TCE atmosphere, and are shown below in Tables 5.4 and 5.5.

Table 5.4. Representative morphologies appearing on phenol-MP-TCE agar plates

No.	color	diameter (mm)	configuration	margin	elevation
1	milky white, semi-transparent	1~2	round	entire	raised
2	milky white to brown, solid	2	round	entire	convex
3	transparent	1~2	irregular and spreading	undulate	flat
4	yellow	2	concentric	undulate	hilly
5	milky white	2	round	entire	drop-like
6	lemon yellow	1	round	entire and smooth	convex

Table 5.5. The numerical abundance of the representative morphologies on serial dilution plates of phenol-MP-TCE agar

	10^{-7}	10^{-6}	10^{-5}	10^{-4}
1	0	50%	60%	30%
2	0	50%	10%	10%
3	0	0	3~5%	30~40%
4	0	0	10%	0
5	0	0	10%	15%
6	0	0	0	15%

According to the principles stated in the material and methods section, isolation and purification were conducted to the colonies on the phenol-MP and phenol-MP-TCE serial dilution plates.

20 colonies were picked from the phenol-MP agar, and 12 colonies from the phenol-MP-TCE agar. Apart from the fast growers that appeared early during the incubation, two or three colonies of similar morphologies were picked at random. The purpose of it was to examine if certain morphology was really of numerical abundance, because colonies of similar morphologies can consist of different bacteria.

Totally 71 pure cultures were isolated from the picked colonies, and they were screened by visual inspection and microscopic observation. The pure cultures of similar morphologies, both on the plate and under microscope, were further examined by REP-PCR to see if they were of the same genotype. Figure. 5.5 below shows two examples of gel-electrophoresis after REP-PCR.

Fig. 5.5. Gel-electrophoresis results of pure cultures of similar morphologies after REP-PCR amplification.

While two lanes (one pure culture per lane) had the same bands at the same positions, they were considered identical, and the two pure cultures were of the same genotype.

After the visual, microscopic and molecular screening, the isolates were found to contain at least 6 dominant species of culturable bacteria. Some of the morphologies introduced in Table 5.2 and 5.4 were found to contain more than one type of bacteria, and some were of the same phenotype but not genotype.

The phenotype characteristics of the dominant bacteria are listed in Table 5.6

Table 5.6. Pure cultures isolated from phenol-grown aerobic granules

Type	Colony morphology	Gram staining morphology
I	light yellow, transparent, 5~6mm, smooth, entire with margin	- thin long rod
II	white, 3~4mm, smooth to wavy margin	- cocci
III	white, dry, flat, 1mm, flowerlike, ragged at rim, white dot at centre	+ thin long rod
IV	pale white, very wet, 5~6mm	+ big short rod
V	transparent, flat, wavy margin, 1~2mm	- small rod
VI	white to light yellow, entire, <1mm	+ purple small cocci

The gram staining morphologies of the isolates are shown below in Fig. 5.6.

Fig. 5.6. Gram staining microscopic observation of pure culture isolated from phenol-grown aerobic granules.

In order to determine if there was any overlapping between the two batches of isolated bacteria from phenol-grown aerobic granules, the dominant isolates listed in Table 5.6 and the isolates obtained by Jiang et al. (2004b) were subject to REP-PCR screening. After comparing their patterns on the agarose gel, the PG03 from Jiang's isolates was found to be identical with type IV culture whether in colony morphology, gram staining

observation or REP-PCR pattern. Therefore, they were considered as the same bacteria, and only one was used in further study of phenol/TCE kinetics.

5.3.2 Phenol degradation kinetics

After the isolation from phenol-grown aerobic granules was completed, the obtained pure cultures were further studied for their phenol and TCE degradation kinetics. The objective was to screen for fast TCE degrader with high growth yield and high TCE transformation capacity. The pure cultures studied included the PG01~PG10 of Dr. Jiang's isolates, and the five types from this batch of isolation. PG 04 was found to cease growing on phenol-MP agar after the incubation with YEPG medium; PG 03 and type III were identical, therefore totally 15 pure cultures were actually studied.

The phenol degradation kinetics of PG01 to PG10 were not determined in this work, as they were already done in previous studies (Jiang et al., 2004b). The newly isolated strains were studied with the same method, for the purpose of comparison.

The phenol degradation kinetics of the isolates are shown in Fig. 5.7 to Fig. 5.11, where both the actual experimental data and the resultant kinetic fitting curves are plotted.

Fig. 5.7. Phenol-dependent specific oxygen consumption rates versus phenol concentrations of type I.

Fig. 5.8. Phenol-dependent specific oxygen consumption rates versus phenol concentrations of type II.

Fig. 5.9. Phenol-dependent specific oxygen consumption rates versus phenol concentrations of type III.

Fig. 5.10. Phenol-dependent specific oxygen consumption rates versus phenol concentrations of type V.

Fig. 5.11. Phenol-dependent specific oxygen consumption rates versus phenol concentrations of type VI.

5.3.3 TCE transformation kinetics

The TCE transformation rates against TCE concentration in the liquid of the isolates are shown in Fig. 5.12 to Fig. 5.24.

Fig. 5.12. TCE kinetics of PG01.

Fig. 5.13. TCE kinetics of PG02.

Fig. 5.14. TCE kinetics of PG03.

Fig. 5.15. TCE kinetics of PG05.

Fig. 5.16. TCE kinetics of PG06.

Fig. 5.17. TCE kinetics of PG07.

Fig. 5.18. TCE kinetics of PG08.

Fig. 5.19. TCE kinetics of PG09.

Fig. 5.20. TCE kinetics of PG10.

Fig. 5.21. TCE kinetics of type I.

Fig. 5.22. TCE kinetics of type II.

Fig. 5.23. TCE kinetics of type III.

Fig. 5.24. TCE kinetics of type V.

Table 5.7. Summary of phenol degradation kinetics

culture	$V_{\max}$ (mg DO g DW ⁻¹ h ⁻¹)	K_s (mg L ⁻¹)	K_i (mg L ⁻¹)	R^2	S^{*b} (mg L ⁻¹)	V^{*c} (mg DO g DW ⁻¹ h ⁻¹)
PG01	1143	36.2	60.5	--	46.8	448.8
PG02	553	7.1	50.6	--	19.0	316.1
PG03	479	12.8	150.1	--	43.8	302.4
PG04	369	70.5	165.2	--	107.9	160.0
PG05	344	45.6	60.5	--	52.5	125.7
PG06	74	3.5	42.0	--	12.1	46.9
PG07	ND ^a	ND	ND	--	--	--
PG08	37	47.2	20.2	--	30.9	9.1
PG09	270	0.4	58.6	--	4.8	231.7
PG10	344	8.3	70.3	--	24.2	203.9
I	78.8	4.14	206	0.68	29.2	61.4
II	1760	68.2	91	0.78	78.8	644.4
III	5525	2484	9	0.77	149.5	161.4
V	41.8	7.19	69.6	0.93	22.4	25.4
VI	83.6	82.9	1990.2	0.76	406.2	59.4

^a Not determined^b S^* is the phenol concentration where the bacterium exhibits its actual maximum phenol degrading rate V^* ^c V^* is the actual maximum specific phenol degradation rate

Table 5.8. Summary of TCE degradation kinetics

culture	$V_{\max}$ (mg TCE g DW ⁻¹ h ⁻¹)	K_s (mg L ⁻¹)	K_i (mg L ⁻¹)	R^2	S^{*a} (mg L ⁻¹)	V^{*b} (mg TCE g DW ⁻¹ h ⁻¹)
PG01	407	45.2	0.38	0.81	4.14	17.8
PG02	13.9	0.98	--	0.81	--	13.9
PG03	1.04	0.86	--	0.43	--	1.04
PG05	15.0	0.67	7.20	0.42	2.20	9.32
PG06	4.78	0.57	--	0.81	--	4.78
PG07	17.1	7.38	2.22	0.64	4.05	3.68
PG08	8.74	0.83	1.86	0.95	1.24	3.74
PG09	3.97	2.11	--	0.92	--	3.97
PG10	16.9	2.01	7.51	0.51	3.89	8.31
I	2.70	0.04	4.42	0.88	0.42	2.27
II	56.3	5.64	2.19	0.77	3.51	13.4
III	33.0	4.21	4.91	0.33	4.55	11.6
V	1433	157	0.14	0.74	4.69	21.1
VI	N.D. ^a	N.D.	N.D.	--	--	--

^a Not detected^b S^* is the phenol concentration where the bacterium exhibits its actual maximum phenol degrading rate V^* ^c V^* is the actual maximum specific phenol degradation rate

Table 5.9. Summary of the transformation capacities (T_C), growth yield on phenol (Y), transformation yield (T_y) and cell hydrophobicity of the isolates.

Culture	T_C (mg TCE g DW ⁻¹)	Y (g DW g phenol ⁻¹)	T_y (mg TCE g phenol ⁻¹)	contact angle (°)
PG01	73.7 (±4.42)	0.63	46.4	58.8 (±7.3)
PG02	114.7 (±8.26)	0.60	68.8	81.7 (±10.2)
PG03	41.6 (±3.00)	0.77	32.0	79.3 (±2.7)
PG05	78.3 (±1.64)	0.73	57.2	45.7 (±11.2)
PG06	52.5 (±2.65)	0.79	41.5	74.2 (±4.2)
PG07	61.9 (±6.07)	ND	--	72.2 (±3.8)
PG08	62.3 (±4.49)	0.75	46.7	51.3 (±10.8)
PG09	83.3 (±6.12)	0.78	65.0	47.3 (±13.1)
PG10	73.4 (±1.02)	0.78	57.3	52.8 (±18.1)
I	20.7 (±3.84)	0.49	10.1	52.4 (±5.3)
II	30.2 (±2.34)	1.22	36.8	33.8 (±4.6)
III	105.0 (±4.69)	0.46	48.3	45.1 (±9.5)
V	103.5 (±10.2)	0.70	72.5	46.4 (±7.8)
VI	26.1 (±7.81)	0.62	16.2	65.9 (±13.3)

5.4 Discussion

5.4.1 Plate count and isolation

The isolation was conducted on four sets of solid media according to the method introduced in the previous sections. As was listed in Table 5.1, the viable count of the bacteria obtained from the total plate count agar plates was 5.2×10^7 per mg VSS of the inoculum, and that on the phenol-MP plates was 2.1×10^7 per mg VSS.

If the counting on total bacteria count agar was viewed as the indication of total viable bacteria number within the granular sludge, and the phenol-MP count as the number of phenol-degraders, it could be inferred that around 40% of the total bacteria were active phenol degraders. The actual percentage could be higher, as phenol is a growth inhibitor. Many colonies may not appear on the phenol-MP plates, not because they couldn't grow on phenol, but because they couldn't tolerate the overlapping stress of phenol toxicity and change of living environment. This can be partly proved by the different lengths of lag phases on the total count and phenol plates (see Figures 5.1 and 5.2, lowest dilution). On the total count agar plates, most CFUs were formed within the first 5 days of incubation, and only modestly increase was observed in the next 100 days or so. However on the phenol plates, a five days lag phase preceded significant colony growth, and most CFUs appeared between 5 and 20 days.

Another factor may also lower the observed percentage of phenol degraders within the granules. Some colonies were found to grow on the top of other colonies on the phenol-MP agar surface, e.g. no. 1, 2 and 3 in Table 5.2, therefore lowered the plate count number.

This might indicate a syntrophic relationship, because the three bacteria were found not to grow alone on phenol-MP agar. Their appearance seemed to be dependent on the growth of another bacterium between their colonies and the agar surface. The colonies under the three bacteria were also isolated, and were found to be identical with the no. 4 in Table 5.2 and no.3 in Table 5.4, through REP-PCR screening. They might degrade phenol to an intermediate which could be utilized by other bacteria, or serve as a base for other bacteria to attach on the agar surface. Another possibility was that the no. 1~3 bacteria were only able to tolerate low concentration of phenol. As introduced in Chapter Three, aerobic granules have complex structures, and some bacteria living in the inner layers of the granules may not be able to tolerate high phenol concentration. The bacteria growing underneath may function as a diffusion barrier through which phenol concentration is much reduced. A third possibility was that these bacteria were predators which fed on the bacterium growing underneath them.

The dominant colony morphologies on the phenol-MP plates are shown in Table 5.3. It could be seen that under different dilutions, the relative abundance of the individual morphologies differed. On the highest dilution plates (10^{-7}), only two morphologies were observed, which made up to 100% of the abundance. However, their abundance on the plates decreased with lower dilution, and on the 10^{-4} plates only constituted less than 5% of the total plate counts. With the decreasing dilution, the diversity of the dominant morphologies increased.

Another set of phenol-MP agar plates receiving the same inoculum were incubated in the dessicator containing 3~5 mg L⁻¹ TCE in the gaseous phase. The purpose of this incubation condition was to study the effect of TCE stress on the phenol-degrading bacteria during the incubation.

Compared to the phenol-MP plates, several differences were observed for the phenol-TCE-MP plates. First, the phenol-MP plates incubated in TCE atmosphere gave the viable count of 6.2×10^6 per mg VSS of inoculum, and no colony appeared on the highest dilution (10^{-7}) plates. Compared to the phenol-MP plates incubated in normal air, a three fold decrease of counting was observed. It could also be seen from Table 5.1 that the plate counts of the 10^{-7} , 10^{-6} and 10^{-5} dilutions were much lower on the phenol-MP-TCE agar than on the phenol-MP agar. Only at the lowest dilution (10^{-4}) did these two sets of agar plates exhibit comparable plate counts. Therefore it could be inferred that the TCE atmosphere exerted stress on the bacteria, especially at higher dilutions where the bacteria density was lower.

Other than the decrease of bacterial count, the exponential phase of the microbial growth also appeared much later on the TCE plates. Without TCE in the air, the sharp increase of plate counts on the phenol-MP plates appeared between day 3 to day 11 of the incubation, while with TCE, the exponential phase started at day 5 and was prolonged to day 25.

Third, the colonies morphologies observed on the phenol-MP-TCE plates exhibited significant differences to those on the phenol-MP plates. The sizes of the colonies on the

TCE plates were generally smaller than those on the phenol plates, especially at higher dilutions. At all dilutions, the dominant morphology on TCE and phenol plates differed. The no. 1 and 2 type of morphologies listed in Table 5.2 were the only ones found on the 10^{-7} phenol-MP plates, however, these two morphologies were only found in small proportions on phenol-MP-TCE plates (10% on the 10^{-5} dilution plates). On the other hand, the no. 6 and no.8 morphology types on the phenol-MP plates, which constituted less than 10% of the total colony count, were dominant on the phenol-MP-TCE plates. The only exemption was the transparent type, No. 4 on the phenol agar and No. 3 on the phenol-TCE plates, which made up to 60% and 40% of the total colony count on the 10^{-4} dilution of the both sets of plates, respectively.

Therefore, the additional stress of TCE in the air did cause change of the incubation results on the phenol-TCE-MP plates. Such changes could be due to the stress of TCE itself or its transformation products, as introduced in Chapter Four. TCE is an organic solvent, and when it is evaporated in the air, microbes in contact with it may be affected by the solvent stress. In this study, the phenol-TCE-MP plates were not sealed, and were incubated in a dessicator containing TCE in the gaseous phase. However the TCE concentration in the air ($3\sim5\text{ mg L}^{-1}$) was not likely to cause severe direct toxicity, as most isolates didn't exhibit reduced TCE degradation rate in this concentration range (refer to Figures 5.11 to 5.23).

The second possible stress is the transformation product toxicity. As discussed in Chapter Two and Four, TCE degradation produces toxic products that can bind to and inactivate cellular materials. Phenol was provided to the phenol-TCE-MP plates as the sole carbon and energy source, and TCE did not support microbial growth. Therefore the colonies appearing on the phenol-TCE-MP plates must be either phenol degraders or oligotrophs. The latter possibility could be ruled out, because all the isolated obtained were able to grow in liquid medium where phenol was supplied as the sole carbon source.

To degrade phenol, the enzyme phenol-hydroxylase must be synthesized, and the same enzyme involuntarily catalyzes the epoxidation of TCE. Therefore, during the growth on

the phenol-TCE-MP plates, the phenol degrading bacteria unavoidably experienced the stress of TCE transformation product toxicity. This additional stress might cause the change of microbial morphology and diversity on the phenol-TCE-MP plates, compare to the phenol-MP plates.

Two kinds of bacteria might be expected to survive better and dominate on the phenol-TCE-MP plates. The first was those which synthesize phenol-hydroxylase of low activity. With lower enzyme activity, the degradation of TCE is slower, and the toxic transformation products are produced more slowly, giving the bacteria time to recover. The other kind was those bacteria with highly efficient phenol-hydroxylase. When phenol is degraded with high efficiency by such bacteria, high growth rate might be achieved, so as to compensate for the loss of cellular activity due to TCE toxicity. The transformation capacity of the bacteria was also important for the survival on the phenol-MP-TCE plates. The bacteria with high transformation toxicity could possibly survive better and grow faster in the TCE environment, and selected as isolates from phenol-TCE-MP plates.

As is shown in Table 5.6 and Fig. 5.6, six bacterial pure cultures were isolated with distinct morphologies and phylogenies. Unfortunately, pure cultures were not obtained for some of the dominant morphologies, as they were found to grow in dependence of other bacteria. A detailed study on the phenol and TCE kinetic properties and TCE transformation capacities of the isolates was then conducted for better understanding of the above discussed phenomena.

5.4.2 Phenol transforming kinetics

The phenol transforming kinetics of the isolates were determined as the phenol-dependent oxygen uptake rates (phenol dependent SOUR) instead of the decreasing rates of phenol concentration, because the former is a more sensitive and accurate method (Watanabe et al., 1996b). From Figures 5.7 to 5.11, it could be seen that the phenol degradation kinetics of all the isolates followed the Haldane's kinetics, that is, reduction of specific phenol uptake rates appeared at higher phenol concentrations.

The five isolates tested in this study could be classified into two kinds according to their saturation and inhibition behaviors. The phenol-dependent oxygen consumption rates of bacteria type I and type V increased sharply under low phenol concentrations, but were immediately and greatly reduced when phenol concentrations further increased. On the other hand, type II, type III and type VI exhibited less abrupt changes with increasing phenol concentrations. The peaks of the phenol dependent SOUR curve appeared at higher phenol concentrations, and were much wider. These differences were the reflections of the different kinetic properties of the individual isolates. To better understand the kinetic behaviors of the isolates, the SOUR data were modeled using Haldane's equation, and the kinetic parameters were obtained.

The kinetic parameters (maximum phenol dependent SOUR, saturation constant and inhibition constant) of 15 phenol degrading pure cultures were summarized in Table 5.7. The results of isolates PG01 to PG10 were obtained from Jiang et al. (2004b), and the R^2 of the modeling was not shown. For the other five cultures isolated in this study, the coefficients of correlation of the simulations were listed as well. The phenol degradation activity of isolate PG07 was too low to be accurately modeled.

The modeling results of the 15 pure cultures showed that there was a wide diversity in the kinetic behaviors of bacteria in the granules. The calculated maximum phenol dependent oxygen uptake rate $V_{\max}$ ranged from 37 to 5525 mg DO g DW⁻¹ h⁻¹, and could be classified to four clusters according to their magnitudes. The first one contained PG08 and type V, which had the lowest $V_{\max}$ values, 37 and 42 mg DO g DW⁻¹ h⁻¹, respectively. PG06, type I and type VI made up the second cluster, with the $V_{\max}$ value ranging between 74 and 84 mg DO g DW⁻¹ h⁻¹. The third group was the most abundant, and had the members of PG02 to 05, 09 and PG 10. Their $V_{\max}$ values clustered around 300 to 500 mg DO g DW⁻¹ h⁻¹. The fourth group had the highest $V_{\max}$ values of all the isolates, which were between 1000 and 6000, and the members were PG01, type II and type III.

The half saturation constant, K_S had a span of three orders of magnitude, ranging from 0.4 to 2484 mg L⁻¹. The clustering of K_S was less obvious. The lowest K_S was that of PG09, while the highest was of type III, which were both singular in the isolates. Others can be grouped into 4 groups: PG06 and type I (K_S around 4 mg L⁻¹); PG02, type V, PG10 and PG03 (K_S from 7 to 13 mg L⁻¹); PG01, PG05 and PG08 (K_S around 40 mg L⁻¹); and type II, PG04 and type VI (K_S from 68 to 83 mg L⁻¹).

The inhibition constant, K_I had the range from 9 to 1990 mg L⁻¹, and the extreme values appeared with type III and type VI. The rest 12 cultures had two clusters of K_I values: 40 to 100 mg L⁻¹ (PG06, PG02, PG09, PG01, PG05, type V, PG10 and type II) and 150 to 200 mg L⁻¹ (PG03, PG04 and type I), the only exemption was PG08, with a K_I of 20.2 mg L⁻¹.

Therefore, whether from the V_{max} , K_S or K_I values of the isolates, it could be seen that the pure cultures isolated from the phenol-grown aerobic granules had a wide diversity of kinetic properties towards phenol. It can be inferred that first, there is a huge pool of kinetically different bacteria in the granules; and second, the isolation process was able to maintain the diversity in the pure cultures. It could also be seen that although less pure cultures were obtained from the second batch of isolation, they had more diverse kinetic parameters than the first batch isolates.

V_{max} is the maximum rate of phenol degradation, and an important parameter in evaluating the application of bacteria. Three of isolates, PG01, type II and type III, had the obvious highest maximum phenol dependent SOUR (V_{max}), 1143, 1760 and 5525 mg DO g DW⁻¹ h⁻¹, respectively. Judged from the K_S values, PG09 had the lowest half saturation constant of all the pure cultures, thence the highest affinity. The bacteria strain with the highest inhibition constant was type VI, and its K_I value reached 1990 mg L⁻¹. However, type III was found to have the lowest inhibition constant of all the cultures (9 mg L⁻¹) and the highest saturation constant, 2484 mg L⁻¹, according to the modeling result.

The strain type III had the most unique kinetic parameters: high $V_{\max}$, high K_S and very low K_I . Put together, these kinetic constants meant that although type III had an obvious high $V_{\max}$, the actual phenol dependent SOUR was much reduced due to the early appearance of phenol inhibition. As shown in Fig. 5.9 that the highest SOUR didn't exceed $200 \text{ mg DO g DW}^{-1} \text{ h}^{-1}$. The reason for this phenomenon might be the possible uniqueness of type III's phenol hydroxylase, or more probably, the error in the simulation process caused by insufficient data points, although the R^2 is not low (0.77). Another strains whose K_I was lower than its K_S was PG08 (K_S 47.2 mg L^{-1} and K_I 20.2 mg L^{-1}), but the discrepancy was not as huge as that of type III.

Therefore, the value of $V_{\max}$ alone is not a sufficient indicator of the phenol degradation ability of the pure cultures. The actual maximum transformation rate, V^* , was calculated as the rate when phenol concentration was equal to S^* . The value of S^* was determined as $(K_S K_I)^{1/2}$. As the phenol degradation kinetics are generally affected by substrate inhibition effect, the specific phenol degradation rate exhibits a peak when plotted against phenol concentration. The peak rate is defined as V^* , the actual maximum transformation rate, and the corresponding phenol concentration where V^* appears is defined as S^* . The actual maximum rates V^* and the phenol concentrations S^* of the isolates were also shown in Table 5.7. The highest V^* was that of type II, $644.4 \text{ mg DO g DW}^{-1} \text{ h}^{-1}$, followed by PG01, $448.8 \text{ mg DO g DW}^{-1} \text{ h}^{-1}$. PG08 had the lowest V^* , which was $9.1 \text{ mg DO g DW}^{-1} \text{ h}^{-1}$. The V^* values of other cultures clustered around 40 to 60, 120 to 160, and 200 to $300 \text{ mg DO g DW}^{-1} \text{ h}^{-1}$, except type V, which had a V^* of $25.4 \text{ mg DO g DW}^{-1} \text{ h}^{-1}$.

S^* is the phenol concentration that corresponds to the actual maximum rate V^* . The lowest S^* was with PG09 (4.8 mg L^{-1}), and the highest was with type VI (406 mg L^{-1}). PG09 also had a moderate high V^* , which means that this pure culture is suitable for the fast degradation of low concentration of phenol. Type VI is a unique bacteria strain, in that it had a far much higher K_I than the rest of the cultures. It could be seen from Figure 5.11 that the curve of type VI was rather flat, and the phenol-dependent oxygen uptake rate was not much reduced even the phenol concentration reached 2000 mg L^{-1} . Therefore

type VI is has high tolerance to the phenol inhibition, and is suitable for treatment of high concentrations of phenol.

The kinetic parameters for whole granules in phenol degradation were: $V_{\max}=1.59\text{mg phenol g SS}^{-1} \text{ min}^{-1}$, $K_S=37.2\text{mg L}^{-1}$, $K_I=461.8\text{mg L}^{-1}$; $V^*=1.02\text{mg phenol g SS}^{-1} \text{ min}^{-1}$ at phenol concentration $S^*=131.0 \text{ mg l}^{-1}$. Because the kinetic parameters of the granules and the pure cultures were obtained through different methods, they (especially $V_{\max}$ and V^*) should be converted to the same unit before comparison. An assumption was made for the conversion, that 2 mol of oxygen is needed for the transformation of 1 mol phenol to catechol, therefore the oxygen consumption rate should be twice the phenol degradation rate.

According to this assumption, the $V_{\max}$ and V^* of phenol granules were calculated to be 65 and 42 $\text{mg O}_2 \text{ g SS}^{-1} \text{ h}^{-1}$, respectively. The $V_{\max}$ of the pure cultures mostly clustered around 300 to 500 $\text{mg O}_2 \text{ g DW}^{-1} \text{ h}^{-1}$, and V^* around 100 to 300 $\text{mg O}_2 \text{ g DW}^{-1} \text{ h}^{-1}$. Therefore, the $V_{\max}$ and V^* of the entire granules were considerably lower than those of the isolated bacteria, but comparable to the cultures isolated in this batch ($V_{\max}$ from 42 to 84 $\text{mg O}_2 \text{ g DW}^{-1} \text{ h}^{-1}$ and V^* 25 to 60 $\text{mg O}_2 \text{ g DW}^{-1} \text{ h}^{-1}$).

The direct comparison (as they were of the same unit) of K_S , K_I and S^* gave different results. The majority of the K_S values of the pure cultures were within the range of 10 to 80 mg L^{-1} , which was comparable to the K_S of granules (37.2 mg L^{-1}). However, the K_I of the granules, 462 mg L^{-1} was well above the K_I values of most pure cultures (40 to 200 mg L^{-1}). Similar was the case of S^* , where most pure cultures had S^* ranging from 10 to 50 mg L^{-1} , the S^* of whole granules was as high as 130 mg L^{-1} .

In summary, compared with the pure bacteria cultures isolated from the granules, the entire aerobic granules had lower specific phenol transformation rate, but retained the high affinity towards the substrate, and was much improved in the resistance to phenol toxicity at high concentrations.

For better comparison of the phenol degradation abilities, the theoretical kinetic curves of all the pure cultures concluded in the next two figures. They were calculated using the kinetic parameters obtained from the modelings.

Fig. 5.25. Phenol dependent oxygen uptake rates versus phenol concentrations of pure cultures PG01 to PG08. (■) PG01; (▲) PG02; (●) PG03; (×) PG04; (---) PG05; (—) PG06; (+) PG08.

Fig. 5.26. Phenol dependent oxygen uptake rates versus phenol concentrations of pure cultures PG09 to PG10, and type I to type VI. (■) PG09; (▲) PG10; (●) I; (×) II; (---) III; (—) V; (+) VI.

5.4.3 TCE transformation kinetics

TCE transformation kinetics of the isolates exhibited even greater diversity than their phenol degradation behaviors. As mentioned in Chapter Two, most studies reported Michaelis-Menton kinetics for TCE transformation. Only a few found that some inhibitory effect might exist when TCE concentration was high (Folsom et al., 1990). For example, the TCE transformation ability of *Ralstonia pickettii* PKO1 was found to be totally diminished when the cells were exposed to saturation concentration of TCE (1100 mg L⁻¹). However in this study, 10 out of the 14 isolates tested exhibited reduced TCE degradation rate within the test concentrations range (0.1 to 40 mg L⁻¹), while others seemed to be unaffected. PG04 stopped growing on phenol after incubation with YEPG (yeast extract-pepton-glucose) medium, therefore was not tested.

Two bacteria strain had the obvious highest V_{max} , PG01 and type V. As was the case of type III in phenol degradation, these two cultures also had very high K_S and very low K_I (45.2 and 0.38 mg L⁻¹ for PG01; 157 and 0.14 mg L⁻¹ for type V). As a result, their actual TCE degradation rates were much reduced below their V_{max} . Other pure cultures that had lower K_I than K_S were PG07 and type II, but the degree was much less than that of PG01 or type V.

Except the PG01 and type V, the other isolates had the V_{max} values between 1 and 56 mg TCE g DW⁻¹ h⁻¹. The lowest were with PG03 (1.04 mg TCE g DW⁻¹ h⁻¹) and type I (2.70 mg TCE g DW⁻¹ h⁻¹). The highest were with type II and type III (56.3 and 33.0 mg TCE g DW⁻¹ h⁻¹, respectively). The V_{max} of whole granules was 0.852 mg TCE g SS⁻¹ h⁻¹ (see Chapter Three), and much lower than those of the pure cultures.

As mentioned above, PG01 and type V had the highest K_S . The pure cultures that had the lowest K_S , and apparently the highest affinity to TCE were type I (K_S 0.04 mg L⁻¹). The K_S values of the rest of the cultures ranged from 0.6 to 7.4 mg L⁻¹, the majority of which clustered around 1 to 2 mg L⁻¹. This is in accordance with the TCE kinetics of the whole granule, whose K_S was calculated to be 1.02 mg L⁻¹.

Four of the isolated tested didn't exhibit substrate inhibition by TCE within the TCE concentration range tested, therefore their K_I was not shown. Other cultures had the K_I of 2 to 5 mg L⁻¹, except PG01 and type V. However, whole phenol-grown aerobic granules didn't exhibit any inhibition by TCE within the tested concentration range. It might be inferred that the structure of aerobic granules could provide protection to the bacteria attached against TCE solvent stress, as was in the case of phenol degradation mentioned above.

The actual maximum TCE transformation rate V^* was also calculated according to the method described above. For the pure cultures that show no sign of inhibition, their V^* was set as their $V_{\max}$. It could be seen that although the modeling results of PG01 and type V were abnormal, they still exhibited the highest V^* of all the cultures (17.8 and 21.1 mg TCE g DW⁻¹ h⁻¹). Other isolates that could transform TCE with high rates were PG02 and type II (13.9 and 13.4 mg TCE g DW⁻¹ h⁻¹). The lowest V^* appeared with PG03 and type I, which were 1.04 and 2.27 mg TCE g DW⁻¹ h⁻¹, respectively. S^* , the TCE concentration where V^* is reached ranged between 1 and 5 mg L⁻¹, with the exemption of type I (0.42 mg L⁻¹).

One unique pure culture was the type VI. As mentioned above, it had a high tolerance to phenol inhibition, and its phenol degradation rate was not the lowest among the cultures. However, this strain was found to have such a low TCE degradation capacity that its kinetic parameters were not estimable. Only under very low TCE concentration (below 0.5 mg L⁻¹) or after long incubation (more than 48 h) could its degradation rate be calculated, therefore its parameters are not shown in the table.

The TCE degradation curves calculated according to the simulated kinetic parameters are concluded in the next two figures, for the purpose of comparison.

Fig. 5.27. TCE transformation rates versus TCE concentrations in the liquid of pure cultures PG01 to PG08. (■) PG01; (▲) PG02; (●) PG03; (×) PG05; (---) PG06; (—) PG07; (+) PG08.

Fig. 5.28. TCE transformation rates versus TCE concentrations in the liquid of pure cultures PG09 to type V. (■) PG09; (▲) PG10; (●) type I; (×) type II; (---) type III; (—) type V.

5.4.4 Combination of phenol and TCE kinetics

Watanabe et al. (1996b) reported that 10 phenol-degrading bacteria pure cultures isolated from continuous and batch cultures could be classified to three kinetic groups according to their K_S and K_I values in phenol degradation. The low K_S group bacteria were all from continuous enrichment, and had the apparent K_S lower than 0.1 mg L^{-1} , K_I lower than 30 mg L^{-1} . The medium K_S group and high K_S group bacteria included the strains from batch cultures, and had the K_I value of 130 and 280 mg L^{-1} , K_S value of 0.15 and 0.3 mg L^{-1} , respectively. One strain was not included in the kinetic groups, as it had a low K_S but extremely high K_I (700 mg L^{-1}). The $V_{\max}$ of the cultures were between 35 and $1040 \text{ mg O}_2 \text{ g DW}^{-1} \text{ h}^{-1}$, with the pure cultures from continuous enrichment exhibiting higher $V_{\max}$ than those from batch cultures.

According to such classifications, the K_S values of the isolated phenol degradation bacteria in this study were well above those of the high K_S group, presumably due to the fact that all the pure cultures were from batch cultures. The K_I and $V_{\max}$ of the bacteria in this study were comparable to those obtained by Watanabe et al. (1996b), with the exemption of type III (extremely high $V_{\max}$) and type VI (extremely high K_I).

Later it was found that the TCE K_S of a pure culture was in positive correlation with its phenol K_S , therefore the three kinetic groups also applied in TCE degradation (Futamata et al., 2001a). The 13 pure cultures studied by Futamata et al. (2001a) showed a TCE K_S range of 1.4 to 105 mg L^{-1} , K_I range of 17 to higher than 200 mg L^{-1} , and $V_{\max}$ range of 2.4 to $53 \text{ mg TCE g DW}^{-1} \text{ h}^{-1}$. They could be likewise classified to three groups: low-, moderate- and high- K_S groups, which had the K_S for TCE of 10 to 60, 100 to 200 and 500 to $800 \text{ }\mu\text{M}$, respectively. The pure culture that had low K_S for TCE also had low K_S for phenol, and vice versa.

The pure cultures obtained in the present study exhibited generally much lower K_S and K_I than the isolated described above, while the $V_{\max}$ values were comparable. A relationship of their phenol K_S and TCE K_S is shown below in Fig. 5.29. Pure cultures PG04, PG07

and type VI are not included in the figure, as either of their phenol or TCE degradation curve was not obtained. Intact phenol-grown aerobic granule was also included.

Fig. 5.29. Relationship of phenol K_s and TCE K_s of the isolated pure cultures and phenol granules.

Figure 5.29a shows all the cultures and granule K_s comparison, while in Figure 5.29b, two extreme values (type III and V) were omitted for clearer observation. It could be seen that no obvious positive or negative relationship was exhibited by the pure cultures' phenol and TCE K_s . Indeed a large proportion of the pure cultures clustered around the low TCE K_s -low phenol K_s area, however some of them exhibited similar low TCE K_s but much higher phenol K_s , while others were the opposite.

5.4.5 Transformation capacities and other characteristics

The transformation capacities, growth yields and transformation yields were further examined as the important parameters in evaluating pure culture activities in TCE co-metabolism. Contact angle was measured as an indication of the cell surface hydrophobicity. The results are shown in Table 5.9.

The transformation capacities (T_C) of the 14 pure cultures ranged from 20 to 115 mg TCE g DW⁻¹. The lowest T_C belonged to type I, type VI and type II, which were 20.7, 26.1 and 30.2 mg TCE g DW⁻¹, respectively. PG03 and PG06 had higher T_C of 41.6 and 52.5 mg TCE g DW⁻¹, while the T_C of PG01, 05, 07, 08, 09 and 10 centered around 70 mg TCE g DW⁻¹. The highest T_C of the isolates was exhibited by PG02, type III and type V, which was above 100 mg TCE g DW⁻¹. The transformation capacity of the entire granules was 10 to 15 mg TCE g DW⁻¹, slightly lower than those of the pure cultures, again confirming the existence of diffusion limitation.

The growth yields (Y) of the pure cultures on phenol were determined for most of the isolates. Y ranged from 0.46 to 1.22 g DW g phenol⁻¹, most of which clustered around 0.6 to 0.8 g DW g phenol⁻¹. Type II had the highest growth yield (1.22 g DW g phenol⁻¹), while type III and type I had the lowest (0.46 and 0.49 g DW g phenol⁻¹). Combining T_C and Y together, the transformation yields (T_y) can be calculated. The lowest T_y belonged to type I and type VI, which were 10.1 and 16.2 mg TCE g phenol⁻¹, respectively. The highest T_y were with PG02 and type V, around 70 mg TCE g phenol⁻¹. Other cultures had the T_y values ranging between 32 and 65 mg TCE g phenol⁻¹.

The contact angles, and thence the hydrophobicities of the pure cultures were measured, after some pure cultures were discovered to exhibit abnormal phenol-TCE kinetics relationships (i.e. high phenol K_S but low TCE K_S , or the opposite). As was discussed in Chapter Three, phenol is a more hydrophilic substance than TCE, therefore the cell surface hydrophobicity (or hydrophilicity) might twist the phenol and TCE kinetics correlations, which were supposed to be only related to the enzyme activities. In other words, a hydrophobic cell surface might preferably take in TCE than phenol, therefore the bacteria will exhibit lower TCE K_S but higher phenol K_S , than could be expected from enzyme kinetics only. On the other hand, a hydrophilic cell surface may cause the cells to exhibit high TCE K_S and low phenol K_S .

The contact angles of the pure cultures ranged from 30 to 80 degrees, the lowest of which was with type II (33.8°), and the highest were with PG02 and PG03 (around 80°). As the

bigger the contact angle is, the more hydrophobic the cells surface will be, it could be expected that PG02 and PG03 were the most hydrophobic of the cultures. However, their phenol K_S were not extremely high among the cultures, nor were their TCE K_S extremely low. Type II, having a very low contact angle, and thence least hydrophobic, should be expected to situate at the right hand side of Figure 5.29b, that is the high TCE K_S -low phenol K_S side, but it was not. Moreover, the extreme points on Figure 5.29b belonged to PG01 (high TCE K_S -low phenol K_S), which had a moderate hydrophobicity (contact angle 58.8°). Other two pure cultures clustered around upper-left region of the graph (low TCE K_S and high phenol K_S), PG05 and PG08 also had the average contact angle values (45.7 and 51.3°). Therefore, at least in this study, the effect of cell surface hydrophobicity on the phenol and TCE kinetics was not significant. The kinetic behaviors of the pure cultures could be expected to be mainly the function of enzyme activities.

5.5 Conclusions

In this chapter, the results obtained in the isolation of pure cultures from granules, and the study of the isolates' kinetic properties was presented. Six pure cultures were obtained from the phenol degrading aerobic granules, one of which was found to be identical to a previous isolate. 15 strains were studied for their phenol and/or TCE degradation kinetics, and their TCE transformation capacity and yield.

Wide diversity was found to exist in both the kinetic behaviors and other physiological properties of the pure cultures. The morphologies of the isolates included gram positive and gram negative cocci, short rods and long rods of different sizes and different cell surface hydrophobicities. Their phenol degradation kinetics exhibited 50 folds different V^* (9 to $450 \text{ mg O}_2 \text{ g DW}^{-1} \text{ h}^{-1}$) in the range of 5 to 410 mg L^{-1} phenol concentrations (S^*). The TCE transformation kinetics were even more diverse, as both Michaelis-Menton and Haldane types of kinetic curves were observed with the isolates tested. The

transformation capacities and yields were within a smaller range, but several strains with high T_C and T_y were identified.

Isolates PG01 and type II were found to have the highest ability to degrade phenol, while type VI was the most tolerant to phenol toxicity. PG01, PG02, type II, type III and type V were the most active in TCE degradation, but type I had the highest affinity to TCE. In terms of the TCE quantity that can be transformed by finite biomass, PG02, type III and type V were most preferable, but type III was not among the ones having the highest transformation yield due to its low growth yield on phenol.

Combining all the characteristics of the isolates, the following pure cultures can be considered as best in TCE co-metabolism application: PG02, for its high TCE V_{max} , moderate TCE K_S and high T_C and T_y , also its high hydrophobicity therefore tendency to form aggregates; type III and V, although without the advantage of low TCE K_S and large contact angle. PG01 and type II could also be considered, as the low T_C of the latter could partially be compensated by its high growth yield.

The diversity exhibited by the isolates obtained from aerobic granules is a great advantage, as it enables the granules to be versatile and adaptive under different operation conditions. Under high phenol concentration, the highly tolerant and high V^* bacteria in the granules can quickly degrade phenol to a non-toxic level, while those bacteria with low K_S are able to completely eliminate it. An indication of this was provided by the possible symbiotic relationship between several pure cultures shown in the isolation procedure. Similarly, the diverse TCE kinetics of the isolated bacteria is an indication of the genetic and functional diversity of the indigenous bacteria population in the granules. With proper enrichment or bioaugmentation technique manipulate the abundance of various bacteria population in the granules, e.g. by exposing the granules to a certain phenol concentration at which a certain bacterium grows best, or by attaching a super phenol/TCE degrading isolate onto the granules, the granular reactor will be able to cope up with all kinds of TCE environment and treatment requirement.

CHAPTER SIX

ALTERNATIVE TCE AND PHENOL DEGRADATION IN AN AEROBIC GRANULAR SLUDGE SBR

6.1 Introduction

In Chapter Three to Chapter Five of this thesis, the granules' formation process and the TCE co-metabolic behavior change during that process, the kinetics, rate limiting factors and the toxic effect, and the bacterial diversity of the phenol-grown aerobic granules were explored and discussed. In this chapter, an SBR reactor using phenol-grown aerobic granules as the biomass was built, and its performance in TCE co-metabolism using phenol as the primary substrate was evaluated.

Various attempts of constructing bioreactors for TCE co-metabolism have been made. In term of reactor type, continuous-flow, dispersed growth reactor, biofilm reactor, membrane bioreactor and multi-stage reactor have been tested. Based on the phase of the target compounds, reactors were designed for chlorinated solvents in the liquid state or the gas state. If classified by the primary substrate used, methanotrophic bacteria, phenol, toluene, and propane degraders have been experimented as the biocatalyst in the reactors. Moreover, mixed cultures and various pure cultures have been tested.

Biofilm reactors were among the first to be applied. In 1989, Strandberg et al. constructed a fixed-film, packed-bed continuous bioreactor utilizing methanotrophic mixed culture to treat TCE and *trans*-1,2-DCE. Methane gas was fed as the carbon source and the chlorinated species were dissolved in water. Strand et al. (1991) and Arvin (1991) also used similar strategies to develop completely mixed biofilm reactors where methane was fed as gas, and TCE (and other chlorinated solvents) was supplied in solution form. Segar et al. (1995) adopted a different operational scheme where phenol was used as the

primary substrate and fed in a discontinuous (sequencing batch) mode, and TCE was degraded continuously.

The bioreactors mentioned above were constructed with microbial consortia as the biological factor. Different pure cultures were also evaluated for their potential of TCE co-metabolism in attached growth mode. *Methylosinus trichosporium* OB3B and its copper-resistant mutant were applied in a sequencing biofilm reactor where methane and TCE were fed separately (Fitch et al. 1996). Although the mutant exhibited considerable higher degradation rate, the reactor was not sustainable due to TCE toxicity. Similarly, when a *Burkholderia cepacia* PR123, a mutant of *B. cepacia* G4 constitutionally expressing the TCE degrading enzyme was fixed on glass and carbon, the enzyme was found to be inactivated by high concentrations of TCE in the vapor phase (Sun & Wood, 1997).

Bacteria of dispersed growth were also adopted in TCE co-metabolic reactor operations. Phelps et al. (1990) used different mixed cultures in a continuous-recycle expanded-bed reactor with crushed glass as the support and methane and propane gas as the primary substrate. Various phenol and toluene degraders, such as *Burkholderia cepacia* G4 (Folsom & Chapman, 1991, Landa et al., 1994), *Pseudomonas putida* F1 (Coyle et al., 1993) and mixed cultures (Shurtliff et al., 1996) were coupled with chemostat reactors for evaluation of their TCE co-metabolism capacity. Continuous operated chemostat reactor was also applied by Wilcox et al. (1995) to degrade TCE in the vapor phase with *Mycobacterium vaccae* JOB5 grown on propane. Other researchers dealing with vapor phase TCE adopted gas lift reactor (Ensley & Kurisko, 1994) and bubble column reactor (Hecht et al., 1995), using phenol as the growth substrate.

As this study focuses on aerobic granules, a form of attached cells, the previous experiments on TCE degradation by mixed or pure culture biofilms are summarized below for purpose of better comparison:

Table 6.1 Comparison of TCE degradation activities by biofilm reactors

Growth substrate	Culture	TCE concentration	Rate constants	Reference
methane	consortium	1 mg L ⁻¹ in liquid	First order: 0.02 min ⁻¹	Strandberg et al., 1989
methane	consortium	0.01~5.1 mg L ⁻¹ in liquid	First order: 0.40~5.4 h ⁻¹	Strand et al., 1991
methane	consortium	0.02~1.2 mg L ⁻¹ in liquid	First order: 0.009 d ⁻¹	Arvin, 1991
propane	consortium	20 mg L ⁻¹ pulse in liquid	Max rate: 80 mg g biomass ⁻¹ d ⁻¹	Phelps et al., 1990
methanol	<i>Methylosinus trichosporium</i> PP358	0.5~3mg L ⁻¹ in liquid	Max rate: 12 mg mg cells ⁻¹ d ⁻¹	Fitch et al., 1996
glucose	<i>Burkholderia cepacia</i> PR123	0.4~2.4 mg L ⁻¹ in the air	Max rate: 0.007~0.715 nmol mg protein ⁻¹ min ⁻¹	Sun & Wood, 1997

A major problem with the reactor design is that TCE degradation is not sustainable without the addition of the growth substrate. The addition of the growth substrate is necessary in three aspects: the induction of the TCE catalyzing enzyme, the replenishment of the reducing energy, and the recovery of cells from TCE degradation product toxicity. Some researchers developed genetically engineered pure cultures which can express the gene encoding the enzymes constitutively without the induction by the primary substrate (Shields & Reagin, 1992; Fitch et al., 1996). However, these pure cultures still need a growth substrate to generate energy, and their purity is difficult to maintain in real applications.

Therefore although pure cultures might have higher specific TCE degradation activities, mixed cultures are preferred in reactor operation for their robustness and sustainability. For simplicity, the growth substrates added into the reactor are usually the primary substrates for the non-specific enzymes. However, as the primary substrate and the co-substrate (TCE in this case) are catalyzed by the same enzyme, they compete for the enzyme's active site, causing the competitive inhibition (Bailey & Ollis, 1986). To avoid this problem, some researchers made use of the kinetic properties of the enzymes, and carefully controlled the concentrations of both substrates in the reactor, so that the

competition would be minimized (Reij et al., 1995). Others designed the reactors with a strategy to temporally or spatially separate the primary and the co-substrate.

For the spatial separation, multi-stage reactor was proposed (Alvarez-Cohen & McCarty, 1991a), where the growth of the biomass on the primary substrate is conducted in one reactor, and the grown and induced biomass is circulated into another reactor for TCE degradation. Alvarez-Cohen & McCarty first constructed a two-stage reactor with methanotrophic bacteria (Alvarez-Cohen & McCarty, 1991b). McFarland et al. (1992) also designed their reactor with the same scheme, with the addition of formate as the supplementary electron donor during the TCE degradation stage. Tschantz et al. (1995) further developed the system by operation four TCE degradation tanks in single-pass and cross-flow modes. A problem with the multi-stage reactor is its complicated construction and operation scheme. Another is what should be done to the biomass having degraded TCE, as it is partially inactivated. To simply waste it would cause an excessive growth substrate consumption, but to recycle it to the growth tank for regeneration would require characterization of the degree of inactivation and regeneration, thus add to the complexity of operation.

An alternative would be to conduct the growth and regeneration of biomass and TCE degradation in one reactor, but in different time spans, i.e. running the reactor in a sequencing batch mode. In a study which was most close to the operation of AGSB reactors, a sequencing biofilm reactor was tested, and phenol was used as the growth substrate (Segar et al., 1995). A precipitous decline in TCE removal was observed after several days of operation, resulting in an unstable reactor performance. The possible reason was that a typical biofilm concentration of 2 g L^{-1} was observed in the reactor (Segar et al., 1995), which might be too low to tolerate the TCE toxicity.

Aerobic granular sludge reactor can achieve high biomass retention and activity in the reactor. An SBR reactor has the advantage of small footprint, easy operation and good biomass-liquid separation. AGSB reactors have mostly been operated in SBR modes, and

by adding a phase of non-aerated TCE co-metabolism, alternative primary substrate and TCE degradation could easily be realized.

The objective of this chapter was to design and construct an aerobic granular sludge reactor, and to operate it in a mode where phenol and TCE were alternatively degraded. The sustainability, performance of the reactor, and characteristics of the aerobic granules during the operation were also studied to evaluate the efficiency and stability of the reactor.

6.2 Materials and Methods

Aerobic granular sludge reactor for intermittent phenol and TCE degradation

The designing of a bubble column reactor operated with aerobic granular sludge, phenol as the growth substrate and TCE as the co-substrate was not an easy task. There were several concerns to be addressed.

First, TCE is an organic solvent. The material of the reactor and any tubing or connectors directly in touch with the reactor content should be compatible with the solvent properties of TCE. Therefore, the reactor material must be either stainless steel, Teflon or glass, as their absorbance of TCE is negligible. Glass was finally chosen as the material, as it is light compared to stainless steel, cheaper than Teflon, transparent (so the reactor content could be seen) and easy to make into different shapes.

Second, phenol and TCE were to be alternatively fed to the reactor. In the phenol degradation phase, oxygen and mixing was provided by aeration. However, aeration should be avoided in the TCE degradation phase, as it would cause TCE loss by air stripping. Therefore, a liquid/gas mixture recirculation was chosen as the means to mix the content and replenish oxygen during the TCE degradation phase.

Third, in addition to the non-aeration in the TCE degradation stage, the reactor content should be kept airtight to prevent possible loss due to leakage. However, during the phenol degradation phase, an unobstructed outlet should be provided for the air outflow. An electrical valve was adopted for the conflicting requirements, whose state of open/close could be controlled by the on/off of the power supply to it. Together with a timer, the valve can ensure that the air can flow out of the reactor during the aeration, and the headspace of the reactor was sealed during the TCE degradation.

Fourth, the aeration of the AGSB reactor is usually provided by air stones, which were not suitable in this case, as they might not be inert to solvents like TCE. A glass sinter disc was fixed at the bottom of the reactor to replace air stones and prevent TCE loss due to adsorption. The pore size of the glass sinter disc was 100~150 μm , and the air bubbles generated were of the size of 1~2 mm, which was comparable to those produced by air stones.

Fifth, feeding of phenol was done by using a peristaltic pump, but the same could not be applied to TCE feeding. The peristaltic pump draws liquid from a container with opening to the outside atmosphere, therefore when the liquid is withdrawn from the container the air flows into the headspace to balance the pressure. However, a non gas-tight container and a changing headspace within the container are not compatible with TCE storage and feeding, because TCE is volatile. The opening on the container will cause leakage of TCE. If the container is made gas-tight, the continuous withdrawn of liquid from it will cause increasing headspace volume and decreasing pressure in the headspace. TCE's volatility means it distributes between the liquid and gas phases, and when the distribution is in balance, the concentrations in the liquid and gas can be described by Henry's law constant. The Henry's law constant of TCE is 0.39, at 25 °C, 1 atm. However, a changing headspace pressure will cause the change in the relevant Henry's law constant, hence the balance of TCE concentration in the liquid/gas phase. A changing and uncontrollable TCE concentration in the feeding liquid is not acceptable. To solve the problem, a syringe pump (*New Era* NE-1600) and gas-tight, glass syringes (25 mL) were selected for TCE feeding. With these, a continuous, timed and controlled TCE feeding

could be achieved, and the syringes could dispense TCE feeding solution without creating headspace.

Sixth, the sampling of the AGSB reactor is usually through stainless steel valve, which is not applicable in this case. If the content of the reactor flows freely out from a valve, TCE dissolved in the liquid will volatilize instantly. However, for sampling of granular sludge from the reactor, an aperture with big enough pore size and a stopper is needed. Therefore, two sets of sampling ports were installed on the reactor: one was a Teflon valve with stopper and 2 mm pore size aperture, for sludge and phenol sampling; the other was a glass tube with a screw cap at the end, for TCE sampling. The screw cap, together with a Teflon-faced septum in it, could ensure the reactor was gas-tight. The sampling was done by puncturing the septum with a gas-tight syringe and drawing out the content without contact with the outside atmosphere.

After careful consideration and comparison, a glass column reactor was designed to meet the requirements stated above. The schematic of the design is shown below in Fig. 6.1, and the detailed specification of the glass reactor can be seen in Fig. 6.2.

Fig. 6.1. Schematic of reactor setup. == Viton tubing, — silica tubing.

Fig. 6.2. Detailed configuration of the glass AGSB reactor.

The reactor was of a cylinder shape, with an inner diameter (I.D.) of 40 mm, and a height of 600 mm. At the top section of the reactor, the inner wall of the reactor was grounded. The reactor was provided with a lid whose outer wall was also grounded. Therefore the reactor could be easily opened and cleaned, meanwhile the airtightness could be ensured while the lid was put on. The lid had an outlet at the top, and the outer diameter (O.D.) of the outlet was 80 mm.

Near the bottom of the reactor a glass sinter disk was fixed, the pore size of which was 100~150 μm . Beneath the sinter disc, the bottom of the reactor was round shaped with another outlet of O.D. 80 mm installed vertically at the lowest point. There were two horizontal outlets of O.D. 6 mm just above the glass sinter disk, of the length 30 mm. A same one was installed 185 mm above the sinter disk, at the left hand side of the reactor. These outlets had the anti-slipping outer wall.

There were five outlets at the right side of the reactor, with the distance of 100 mm between each other. They were fitted with Teflon valves for withdrawn of water samples, and the aperture was big enough for particles around 1 mm diameter. Six outlets are placed at the front, at the same level of those at the right. They were made by melting the bottom of a 2 mL HPLC sample bottle (Waters) and sintering it with the reactor. The HPLC sample bottles were fitted at the necks by 9 mm screw caps (*La-Pha-Pack*, with centre holes and PTFE coated septa) to seal the outlets.

The tubing used for any parts in direct contact with the reactor content was of Viton material (Masterflex), which was solvent-tolerant and inert, and could be used with common peristaltic pump and pump heads (Masterflex). The transfer lines that needed Viton tubing were the TCE feeding line from the syringes to the reactor bottom, the recirculation line, the aeration line from the one-way valve to the glass sinter disc, the air outflow line from the reactor top to the electrical valve, the phenol feeding line from the pump head to the middle of the reactor and the effluent line from the middle to the pump head. For the other lines, i.e. from air pump to the one-way valve, from the electrical valve to the exhaust, from phenol storage tank to the pump head and from the pump head

to the drain, silica tubing was used. Those connectors used together with Viton tubing were of either stainless steel or brass, and those with ordinary silica tubing were plastic.

The configuration of the installed glass reactors could be seen in Fig. 6.3 below.

Fig. 6.3. Reactor setup, sampling ports and syringe pump of the glass AGSB reactors.

Operation conditions of the phenol-TCE AGSB reactor

Three AGSB reactors were used in the TCE-phenol alternative degradation operation, one as the control where no TCE was added and the other two received TCE dosages (TCE 1 and 2 as duplicates). The operation volume of the reactors was 500 mL each, and the volumetric exchange ratio was 0.5. After each cycle half of the reactor's working volume was exchanged. The aeration rate during the phenol degradation phases was kept at $2.5\sim 3\text{ L min}^{-1}$ for every reactor. The seed sludge was the mature granules taken from 2 L lab scale AGSB reactors described in Chapter Three. The granules were harvested from the three reactors, mixed and washed with buffer. The MLSS was estimated and the granules were put in the small glass reactors at a concentration comparable to the MLSS in the bigger reactors.

The three reactors were operated in exactly the same sequencing batch mode, the cycle time of which was 4 h. One cycle could be divided to several phases: aeration-phenol degradation phase; recirculation-TCE addition and degradation phase; aeration-residue TCE elimination phase; settling phase; effluent withdraw phase and phenol feeding phase. The length of every phase was: 120, 105, 5, 5, 3, 2 min, respectively.

The first two hours of the operation cycle were the phenol degradation stage where aeration was provided and the electrical valve was switched on. Previous study described in Chapter Three on AGSB reactor showed that at the initial phenol concentration of 250 mg L^{-1} , the influent phenol could be degraded within 45 min. The reasons for providing 2 hours of aeration were mainly for the maintenance of the granule structure, as certain shear force was found to be essential to granule integrity, and to achieve high oxygen concentration in the mixed liquor.

The phenol loading to the reactor and the composition of the influent were the same as those described in Chapter Three and Four. Tap water was used to prepare the phenol feeding solution.

After two hours of aeration and phenol degradation, the air pump and the electrical valve were switched off. TCE feeding was immediately commenced by the syringe pump (New Era, NE-1600). The pump was timed using its internal timer to deliver 2 mL TCE feeding solution every 4 h. The feeding rate was 1.0 mL min^{-1} and the feeding time was 2 min. TCE feeding solution was prepared in the same way as the TCE saturation stock solution, but in larger (125 mL) serum bottles. Syringes were loaded by drawing the TCE solution from the serum bottles through a glass tube (I.D. 3mm) fitted at the tips of the syringes. As the syringes used were of the volume 30 mL, they were loaded every two days. For the control reactor, the content in the respective syringe was replaced by 2 mL of distilled water.

As soon as the TCE feeding began, the recirculation pump was turned on. Air/liquid mixture was drawn from near the surface of the reactor content and pumped into the bottom of the reactor. The aims of the recirculation were first to enhance TCE mass transfer in the reactor and provide more contact between biomass and TCE, and second to replenish oxygen into the aqueous phase. 2 mL of TCE was fed each cycle, which equals to 2.2 mg and 0.018 mmol. To completely mineralize the TCE, 0.027 mmol O_2 , which equals to 0.87 mg is needed (see Chapter Two). Assuming the reactor content had a dissolved O_2 concentration of 8 mg L^{-1} , the total oxygen in the reactor was 4 mg, which was more than adequate for TCE mineralization. The excessive dissolved oxygen (DO) could be used for biomass respiration. The continuous recirculation of air/liquid mixture could also replenish oxygen from the air should the DO became insufficient.

At the end of the TCE degradation phase, the recirculation pump was turned off and the air pump was on. A short aeration period of 5 min was provided to the reactor to blow off any possible TCE residue to the exhaust. Because TCE is potential hazardous to human health, it was not intended to release TCE into the open atmosphere. If any TCE residue was in the liquid, withdraw of the effluent to the drain would cause it to evaporate to the air. Therefore the short aeration phase was added to ensure any un-degraded TCE would leave the aqueous phase before effluent withdraw phase began.

5 min of settling period followed the aeration. The seed sludge used for the TCE—phenol alternative fed AGSB was aerobic granules taken from stably operated lab scale AGSB. Therefore the short settling time (5 min) was used throughout the test. However, it was longer than the settling time of the reactors from which granules were obtained (2~3 min). The reasons for this were that TCE degradation preferred higher biomass concentration, and the change of environment of the granules might require less harsh operation conditions.

The electrical valve on the air outflow line was opened during the aeration, settling, withdraw and influent filling phases.

Sampling and analytical methods

Three kinds of samples were obtained from the reactor: liquid sample for biomass and phenol analysis; liquid sample for dissolved TCE analysis; and gas samples for gas-phase TCE analysis.

The first kind of samples was taken by simply turning on the Teflon valves and collecting around 25 mL liquid outflow, during the phenol degradation stages. The liquid TCE samples were obtained from the septa on the screw caps by a 1 mL gastight syringe (SGE). 0.5 mL of liquid sample was drawn out and injected to a 2 mL GC sampling vial that had already been sealed. 50 μ L headspace of the vial was injected in to gas chromatography and the TCE concentration was calculated as the total mass of TCE in the vial divided by the liquid volume (0.5 mL). The gas phase TCE concentration was also determined by GC, but the sampling was done by directly drawing 50 μ L gas phase sample from the screw caps that were installed above the liquid content level in the reactor. The tests were duplicated.

The configuration and operation condition of the gas chromatograph were as described in Chapters Three and Four. The analysis of the sludge characteristics were according to APHA (1998), and the parameters measured were MLSS, MLVSS, SVI, SOUR, size and morphology. In the measurement of SVI, 10 mL cylinder was used instead of the 100 mL

one, because of the smaller scale of the reactor. The length-diameter ratios were determined to be the same for both kinds, therefore the SVI obtained could be considered as comparable. MLSS and MLVSS samples were duplicated but SVI and SOUR measurements were not.

6.3 Results

6.3.1 Abiotic test

Before the AGSB reactor was applied in TCE co-metabolic degradation, it was first operated with identical conditions with tap water instead of granules. The purpose was to see if the conditions were suitable for the application, e.g. that the reactor was gas-tight so that no TCE was lost due to leakage; the aeration rate was high enough to produce dissolved oxygen and mixing; the recirculation was adequate to ensure TCE mass transfer when aeration was turned off. The TCE concentrations in the reactor's two phases, liquid and headspace, during a cycle time are shown below.

Fig. 6.4. Change of TCE concentration in the reactor liquid and gas phases without biomass addition. (■) liquid phase; (▲) gas phase. The data represent the average of duplicated samples.

The TCE concentration in the liquid phase had been fluctuating around 1 mg L^{-1} after the injection of 2 mL TCE saturated feed solution. In the headspace, TCE concentration increased from 0.75 mg L^{-1} at the injection time to 2 mg L^{-1} 10 min after, and was stable afterwards. At 120 min after the injection, aeration was turned on for 5 min to purge the reactor content. Sampling at 130 min showed no TCE residue in either liquid or headspace phase.

6.3.2 Phenol degradation of in reactor

The phenol concentration profile in the three reactors during one degradation cycle is shown below in Figures 6.5 and 6.6.

Fig. 6.5. Phenol degradation cycle on day 1 of reactor operation. (■) TCE reactor 1; (●) TCE reactor 2; (▲) control reactor. The data represent the average of duplicated samples.

Fig. 6.6. Phenol degradation cycle on day 41 of reactor operation. (■) TCE reactor 1; (●) TCE reactor 2; (▲) control reactor. The data represent the average of duplicated samples.

From Figures. 6.5 and 6.6, it could be seen that the phenol provided to the reactors could be degraded within 20 min of the cycle beginning, both on day 1 and day 41 of the operation. On day 1, the three curves of degradation were almost identical, while on day 41, some difference was observed that the degradation rate in the control reactor (no TCE addition) seemed lower.

The phenol concentrations in the influent and discharge were regularly monitored. The measured initial concentration of phenol was between 200 and 300 mg L⁻¹, averaged around 250 mg L⁻¹. Phenol concentration in the discharge liquid was universally below detection limit.

6.3.3 TCE degradation in the reactor

Figure 6.7 shows the fluctuation of the influent and effluent TCE concentrations in the reactor operation. The samples of initial TCE concentrations were taken 5 min after TCE injection at the beginning of a TCE degradation cycle, when the content of the reactor

was fully mixed, as was shown in Fig. 6.4. For effluent concentrations, samples were taken at the end of a cycle right before the recirculation stopped.

Fig. 6.7. Initial and effluent TCE concentrations during the reactor operation. (■) Initial concentration in the liquid phase; (▲) Initial concentration in the gaseous phase; (●) effluent concentration in the liquid phase; (×) effluent concentration in the gaseous phase. The data represent the average of duplicated samples.

During the six weeks of reactor operation, the initial TCE concentrations, whether sampled from the liquid phase or from the gaseous phase of the reactor, remained stable. The concentrations in the liquid fluctuated between 1.0 and 1.4 mg L⁻¹, and those in the headspace were slightly higher (around 1.7 mg L⁻¹).

The effluent concentrations were sampled at the end of the cycles, before the aeration was turned on. The effluent concentrations in the liquid phase were mostly below detection limit. However, the concentrations in the headspace exhibited a periodical fluctuation, between 0 and 0.4 mg L⁻¹.

A full cycle of TCE degradation can be seen in Fig. 6.8. The cycle lasted 120 min, during which 10 samples from both the liquid and the headspace were obtained. The first sample was taken 5 min after the injection, which showed TCE concentrations of 0.63 and 0.89 mg L⁻¹ in the liquid and headspace, respectively. The apparent TCE concentrations increased to 1.43 and 2.15 mg L⁻¹ at 10 min, as evident from the second sample. Both TCE concentrations in the liquid and the headspace continued to decline in the next 90 min, and at 100 min they were essentially below detection limit. Aeration was turned on at 105 min, and the remaining TCE (if any) was purged.

Fig. 6.8. TCE degradation cycle. (■) concentration in the liquid (▲) concentration in the headspace. The data represent the average of duplicated samples.

6.3.4 Biomass characteristics in the reactor

The biomass in the control reactor and two TCE-degrading reactors (termed TCE) was characterized during the operation duration. The changes of suspended solids concentration and the sludge volume index of the biomass are shown below in Fig. 6.9. The results from the two TCE reactors were averaged.

Granular sludge was inoculated in the three reactors, and the starting SS concentrations were kept the same, around 3.2 g L^{-1} . VSS was 64% of SS, and the SVI of the seed granules was 35 mL g SS^{-1} . The SOUR and endogenous respiration rate were 104 and $14.3 \text{ mg O}_2 \text{ g VSS}^{-1} \text{ h}^{-1}$, respectively. The three reactors were operated for 10 days before TCE was added, for the purpose of acclimation and stabilization of the granules to the small glass reactors. The operation mode was identical to that would be used in the TCE degradation operation.

Fig. 6.9. Change of SS concentration and SVI value in the control and TCE reactors. (●) SS of TCE reactor; (■) SS of control reactor; (×) SVI of TCE reactor, (▲) SVI of control reactor.

After 10 days of operation, the SS concentrations rose to 5.3 and 6.7 g L^{-1} in the TCE and control reactors, respectively. The VSS to SS ratio increased to 89%, but SVI in both reactors also rose to 100 and 70 mL g SS^{-1} (TCE and control respectively). The TCE injection was then initiated, and the TCE-phenol alternative degradation operation was conducted.

The test lasted for 41 days. SS concentrations in the two kinds of reactors were similar for the first 25 days, which fluctuated between 4 to 6 g L⁻¹, averaged around 5.3 g L⁻¹. However, in the last 15 days of the operation, the SS in the control reactor showed a decreasing trend, compared to that in the TCE reactors, which remained stable. The SS at the end of the operation were 4.5 and 5.3 g L⁻¹ for the control and the TCE reactor, respectively.

The SVI in both reactors dropped sharply in the first week of operation to around 30 mL g SS⁻¹, and were almost identical in the next week. However, from day 14, a steady increase of SVI in the control reactor was observed, which reached the highest value of 97 mL g SS⁻¹ at day 31 and decreased to 60 mL g SS⁻¹ in the next 10 days. In the meantime, the SVI in the TCE reactors was on a steady decline, and dropped to 26 mL g SS⁻¹ at the end of the operation.

Fig. 6.10. Change of SOUR and granule size during the operation. (■) SOUR in the TCE reactor; (▲) SOUR in the control reactor; (+) size in the TCE reactor; (×) size in the control reactor.

Figure 6.10 shows the change of the specific oxygen utilization rate (SOUR) on phenol and the granular sludge size of the TCE and control reactors. The sludge used as inoculant had the SOUR of $104 \text{ mg O}_2 \text{ g VSS}^{-1} \text{ h}^{-1}$ and the size of $950 \text{ }\mu\text{m}$. After 10 days of acclimation, the SOUR rose to around $200 \text{ mg O}_2 \text{ g VSS}^{-1} \text{ h}^{-1}$, and the mean size dropped to around $600 \text{ }\mu\text{m}$ at the beginning of TCE degradation operation.

Before day 10, the SOUR in both reactors increased in similar fashion, from around 200 to 564 (control) and 475 (TCE) $\text{mg O}_2 \text{ g VSS}^{-1} \text{ h}^{-1}$. However, from day 10 to the end of the operation, SOUR in both TCE and control reactors were dropping, more significantly in the TCE reactors than in the control. At the end, the SOUR in the TCE reactor was $93 \text{ mg O}_2 \text{ g VSS}^{-1} \text{ h}^{-1}$, and that in the control reactor was $271 \text{ mg O}_2 \text{ g VSS}^{-1} \text{ h}^{-1}$.

The size and morphology of the biomass was determined by Image Analysis (refer to Chapter Three). At day 0, the means size of the biomass was 624 and $588 \text{ }\mu\text{m}$ in the control and TCE reactors, respectively. Subsequently this parameter showed different trend in the two reactors, except in the first week when it decreased to 563 (control) and $537 \text{ }\mu\text{m}$ (TCE). Biomass size in the control reactor was on a continuous decline, and reached $383 \text{ }\mu\text{m}$ at the end of the operation. While in the TCE reactor, the size remained stable for the first 25 days, and showed an increase in the last 2 weeks of operation. The mean size in the TCE reactor at day 41 was $522 \text{ }\mu\text{m}$, comparable to that at day 0.

Change of morphologies of the granules in both reactors is shown in Fig. 6.11. The morphologies in the TCE and control reactors were similar at day 0, therefore only one picture is shown. During the operation, granules in both TCE and control reactors showed sign of disintegration, e.g. after 10 days of acclimation the mean size of the granules at day 0 was smaller than that of the seed granules. However, in the TCE reactor, the biomass retained the round and entire shape at the end of the operation, and smaller granules were observed, probably formed from the disintegrated biomass particles. In the control reactor, the biomass continued to break up and at day 41, hardly any granules could be observed in the picture.

Fig. 6.11. Change of biomass morphologies in both TCE and control reactors

6.4 Discussion

6.4.1 Abiotic test of reactors

The reactor was first tested with 500 mL tap water as the content, with no biomass addition. The test duration was 120 min, the same as that would be used in phenol-TCE alternative degradation application. The recirculation pump was turned on in the test, and the gas outflow valve was turned off.

As both the TCE concentrations in the liquid and headspace phases were stable throughout the 2 h operation session, it could be inferred that the reactor was gas-tight. There was no significant leakage at the tubing-reactor connections, nor at the Teflon valve or the screw cap sampling ports. The design and setup of the reactor were proved to be sufficient for the application, and the possible TCE loss could be solely attributed to biological degradation.

The TCE concentration in the liquid phase was immediately stabilized after injection, but the same effect could only be observed after 10 min of recirculation in the headspace phase. This was probably due to the fact that the TCE feed solution was directly injected into the bottom (liquid phase) of the reactor, and the dilution effect was instant. However, the diffusion of TCE from the liquid to the headspace phase took some time (in this case 10 min), so the equilibrium was not immediately observed.

It was introduced in Chapter Two that at 25 °C, the equilibrium of TCE between the water and gas phases could be described by the dimensionless Henry's law constant of 0.39. That is to say, the equilibrated TCE concentration in the gas should be 0.39 of that in the water. However, in this study the measured TCE concentration in the headspace was higher than that in the water. The possible reason for this phenomenon was that, the liquid phase in the reactor was constantly stirred by the recirculation from near the surface to the bottom, but the mixing of the headspace with the liquid was poor. Therefore it was not an exact well-mixed system, and the constant probably didn't apply

very well. In the subsequent study, the TCE mass in the reactor would be represented by its concentrations in both phases, instead of that in the liquid alone.

6.4.2 Phenol degradation

The influent phenol concentration was kept around 500 mg L^{-1} , so the initial concentration in the reactors was 250 mg L^{-1} . In Chapter 3, it was showed that the acclimated sludge in the reactors could degrade the added phenol in 45 min. In these smaller reactors, the phenol degradation was done in 20 min, which could be attributed to the increased biomass concentration and high SOUR of aerobic granules.

TCE degradation was reported to have detrimental effect on biomass, and lowered the primary substrate degradation ability. However in this operation, reactors TCE 1 and 2 still showed remarkable phenol degradation activity after 40 days of TCE addition. The phenol degradation curves were comparable in the three reactors, including the control without TCE addition. Therefore, the phenol-TCE alternatively fed AGSB reactors were proved to be sustainable.

An important reason for the sustainability of the reactors is that, they were designed to operate far below the transformation capacity of the granules. Using an average biomass concentration of 5 g SS L^{-1} and the T_C of $10 \text{ mg TCE g SS}^{-1}$, each reactor was calculated to be able to degrade 25 mg TCE before the T_C was spent, while the daily dosage of TCE to the reactors was 12 mg TCE. Moreover, 250 mg L^{-1} phenol was provided each cycle to reactivate the granules from TCE TPT. The frequently switching between TCE degradation/inactivation and phenol degradation/reactivation, and the low TCE loading during each degradation phase contributed to the sustainability of the reactors. Other researches also confirmed the advantage of the SBR mode (Shih et al., 1996).

6.4.3 TCE degradation

Figure 6.7 shows the fluctuation of the initial and effluent TCE concentrations in both reactors. The concentrations in the liquid were stabilized between 1.0 and 1.4 mg L^{-1} , and

those in the headspace were around 1.7 mg L^{-1} . The concentrations detected from the gas phase samples were higher than those from the liquid phase in the reactor, which was contradictory to the Henry's law, as it states that at 25°C , the equilibrated TCE concentration in the gas phase should be 0.39 fold of that in the aqueous phase. The reason for such a phenomenon might be that the samples for the initial TCE concentrations were taken at 5 min after the cycle beginning. During this 5 min, the recirculation pump was on, and the content in the reactor was mixed by pumping liquid from the upper surface to the bottom. By such mixing, the liquid content of the reactor was constantly disturbed and TCE dissolved in it was driven out to the gaseous phase. Meanwhile, the solution of TCE from gaseous phase back to the liquid was not as efficient, as the headspace was not pumped or circulated to the liquid. Therefore this reactor is not a well-mixed system, as described by Henry's law, causing the deviation.

The effluent concentrations in the liquid phase were mostly below detection limit, implying that almost all TCE in water was removed. The effluent TCE concentration sampled from the headspace of the reactor showed fluctuations, the highest of which was around 0.4 mg L^{-1} . This could also be attributed to the inadequate mixing between the gaseous and aqueous phases, resulting in occasional residue TCE in the headspace. However, most time of the operation, the reactor had achieved total TCE removal from both the liquid and gas phase.

A sample of TCE degradation cycle was shown, confirming the TCE removal efficiency of the reactors (Fig. 6.8). Except in the first 10 min of the cycle, the TCE concentrations continued to drop in both the liquid and the headspace phases, more sharply in the headspace. While in the first 10 min, the concentrations increased from 0.63 and 0.89 mg L^{-1} to 1.43 and 2.15 mg L^{-1} in the liquid and gaseous phases, respectively. The fluctuation of TCE concentration in the headspace was more prominent than that in the liquid, indicating that the volatilization and solution of TCE from the liquid to the headspace took place. The reactor and the process were able to completely remove TCE in the influent, as was evident by the TCE concentrations at the end of the cycle, which were below detection limit.

The results achieved in this prototype glass reactor utilizing phenol as the growth substrate and aerobic granules as the biomass have shown promise for a bigger scale TCE co-metabolism process. The target TCE concentration is likely to be within the range of 0.1 to 20 mg L⁻¹ in the liquid, taking into consideration the K_S of the granules and pure cultures. Phenol could be provided at concentrations much lower than 250 mg L⁻¹ to reduce growth substrate consumption. Materials other than glass could be adopted to construct the reactor, due to the fragile nature of glass. Better mixing and smaller headspace should be considered for the future reactor design, as they have been found to be problematic in this study.

6.4.4 Change of biomass characteristic

The inoculum for the TCE-phenol alternative degradation reactors were granules that had been cultivated in 2 L, 5 cm inner diameter reactors and stored under 4 °C for 2 months. Therefore, before the TCE degradation test was began, a 10 day acclimation period was provided. The stored granules were inoculated to the three reactors at similar concentration, and the operation conditions were set the same as those that would be used in the TCE degradation test, i.e. alternative aeration and recirculation. The purpose of the acclimation was to reactivate the granules from the low temperature storage and to acclimate them to a different reactor configuration (500 mL working volume, 4 cm inner diameter).

After 10 days of acclimation, the average SS concentration nearly doubled (from 3.2 g L⁻¹ to 6.0 g L⁻¹), and the VSS/SS ratio increased from 64% to 90%. Together with the change of SOUR from 104 to 203 mg O₂ g VSS⁻¹ h⁻¹, these phenomena strongly indicated that the reactivation of the granules from cold storage was successful. The granules fully recovered their active biomass fraction and phenol degradation ability. However during this period, the SVI value increased by 1.5 folds, and the mean size dropped from 950 to around 600 µm, which might suggest that the change in the reactor configuration had some negative effect on granule integrity and settling ability. With the continuance of

operation, SVI dropped to below that of the seed granules at day 7, and size stabilized, indicating the acclimation of granules to the smaller reactor after longer time.

After TCE degradation was commenced, the changes of SS in the two reactors were similar, until day 25, from when SS in the control reactor began to drop. The deviation of SVI values in the control and sample reactors appeared earlier, and from day 15 the SVI of the control continued to increase. Accompanying these phenomena, the granule morphology in the control reactor also showed dramatic change, that the biomass was mainly composed of flocs and scarcely any integrated granules could be observed from Fig. 6.11. Therefore, the granules in the control reactor began to deteriorate around 2 weeks from the start of the operation, and the decreased settling ability caused the washed out of biomass and hence the drop in SS.

Concurrently, the SOUR in the control reactor remained stable before day 22, after an increase from around 200 to 564 mg O₂ g VSS⁻¹ h⁻¹. From day 10 to day 41, the SOUR in the control reactor declined slightly. During this period, SOUR in the TCE reactor showed a more prominent decrease, and at day 41 was even below that of the seed granules. Previous batch studies on the TCE toxicity (Chapter Four) showed that when small amount of TCE was applied to the granules, the phenol-dependent SOUR could be improved. Such stimulation effect was even sustainable throughout three phenol additions, 72 h. However from the reactor results, it seemed that repetitive TCE addition had some accumulative toxic effect, and eventually lowered the SOUR of the granules that had been degrading TCE.

Although the control reactor exhibited higher SOUR, the other characteristics of its biomass were not satisfying, especially the biomass size and morphology. The mean size of the biomass continued to decrease from day 0. From Fig. 11, at day 41, the sludge of the control reactor was almost totally comprised of flocs, while that in the TCE reactor retained intact granular sludge of various sizes.

Other researchers had observed the morphology change of activated sludge in a TCE-phenol alternatively fed SBR (Ayala-del-Río, 2004). It was found that compared to reactor receiving phenol only, the phenol-TCE reactor produced biomass of larger size and apparent better flocculation. The flocs in the phenol-TCE reactor showed sign of auto-aggregation. On the other hand, the phenol degrading sludge receiving regular TCE dosage exhibited lower but more stable phenol-hydroxylase activity. These findings coincide with the result obtained in this chapter, that granules in the TCE reactor were better preserved of their structural integrity, and showed lower phenol-dependent SOUR than the control reactor.

6.5 Conclusions

A glass SBR reactor was designed to facilitate alternative phenol and TCE degradation by phenol-grown aerobic granules. The reactors were tested for the possible leakage and adsorption, and no significant loss of TCE due to these abiotic factors was discovered. The reactors were inoculated with phenol-grown aerobic granules cultured in larger 2 L reactors, and 10 days of acclimation period was provided, followed by 6 weeks of TCE-phenol alternatively fed test.

The phenol degradation in all three reactors (two TCE, one control) was found to be complete, and the phenol in the influent was degraded within 30 min. The two TCE-fed reactors also showed good TCE degradation activity, and were able to completely remove the fed TCE within the cycle time limit. The effluent TCE concentration in the liquid was essentially below 0.1 mg L^{-1} , while that in the headspace at the end of the cycle showed fluctuation between 0 and 0.4 mg L^{-1} . The residual TCE in the headspace was possibly caused by the insufficient mixing condition. However, the reactor was still able to achieve 90~95% TCE removal efficiency during the entire test duration, including TCE present in the headspace.

The granules in the TCE reactors exhibited lower SOUR and SVI value, and higher SS and size than that in the control reactor. These differences were probably caused by TCE addition, which stimulated the integration of biomass, but lowered the phenol degradation ability. The granules in the TCE reactors were able to fully retain their structural integrity, round shape, and good settling ability after 6 weeks of operation. However, granules in the control reactor almost totally disintegrated and almost no granular structure was observed.

The reactor and process designed were proven to be capable of alternative phenol and TCE degradation. The process was sustainable and efficient.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

In the previous four chapters (Chapter Three to Chapter Six) of this thesis, the results obtained in several aspects of the co-metabolism of TCE by phenol-grown aerobic granules were presented. They include:

- Granulation with phenol as the growth substrate
- Change of biomass' TCE degradation activities during granulation
- TCE and phenol degradation kinetics of the mature granules
- Transformation capacity and mineralization potential of the mature granules
- Rate limiting factors in TCE co-metabolism by the granules
- Direct and transformation product toxicity of TCE co-metabolism
- Phenol degradation by granules after TCE transformation
- Diversity of bacterial community within granules
- Diversity of TCE and phenol transformation kinetics exhibited by the pure cultures isolated from mature granules
- Selection of the suitable isolates for fast and sustainable TCE co-metabolism
- Designing of a granular sludge reactor for alternative TCE and phenol degradation
- Operation and sampling of the reactor and TCE degradation monitoring
- Observation of biomass characteristics during TCE-phenol alternative degradation

Researches conducted on the above aspects have generated many interesting results, which can be concluded as follows:

7.1.1 Cultivation of aerobic granules using phenol as the growth substrate

Aerobic granules were successfully cultivated, using phenol as the sole carbon and energy source, and fully acclimated phenol-degrading activated sludge as the inoculum, after 4 months of granulation operation.

During granulation, the mean size of the biomass increased from 50 to 600 μm , and the SVI dropped from 320 to 30 mL g SS^{-1} . Compared to the seed sludge, the specific phenol degradation activity (SOUR) and TCE transformation activity (T_r) of the mature granules were almost retained, while TCE transformation capacity (T_C) was reduced to half.

7.1.2 Kinetic parameters, transformation capacity and mineralization potential of mature granules

The kinetics of stable granules in TCE and phenol degradation was determined. The parameters for phenol degradation were: $V_{\max}=1.59 \text{ mg phenol g SS}^{-1} \text{ min}^{-1}$, $K_S=37.2 \text{ mg L}^{-1}$, $K_I=461.8 \text{ mg L}^{-1}$. The kinetic parameters for TCE transformation were: $V_{\max}=0.0142 \text{ mg TCE g SS}^{-1} \text{ min}^{-1}$, $K_S=1.02 \text{ g L}^{-1}$.

The transformation capacity was determined to be $0.011 \text{ g TCE g SS}^{-1}$ without the addition of exogenous electron source. Almost total mineralization of TCE was achieved by the granules (2.8 mol Cl^- released by 1 mol TCE degraded).

7.1.3 Effect of additional electron source and biomass size on TCE degradation

Exogenous electron sources didn't have significant effects on the instant TCE transformation rates, and could increase the transformation capacity by 20~30%.

Diffusion limitation was probably the most important factor limiting the TCE and phenol degradation rates by the granules, as the mean size of the biomass was found to significantly and negatively affect TCE and phenol degradation activities.

7.1.4 Direct and indirect toxicity of TCE

The solvent stress and induction effect of TCE were not observed with the aerobic granules.

The phenol-grown aerobic granules had a unique response to the TCE turn-over dependent toxicity, in that small amounts of TCE transformation could enhance the phenol uptake ability of the granules. Such effect was sustainable.

When higher dosage of TCE was applied to the granules, the subsequent phenol dependent SOUR decreased in a linear fashion, but not diminished even when the transformation capacity was spent. TCE degradation didn't reduce the granules' resistance to phenol toxicity.

7.1.5 Isolation of pure phenol degrading cultures from the granules and identification of their phenol/TCE degradation activities

The isolation of pure phenol degrading bacteria from granules was conducted. Six pure cultures were obtained from the phenol degrading aerobic granules, one of which was found to be identical to a previous isolate.

15 strains were studied for their phenol and/or TCE degradation kinetics, and their TCE transformation capacity and yield. Wide diversity was found to exist in whether the kinetic behaviors or other physiological properties of the pure cultures.

Phenol degradation was found to be following Haldane kinetics for all the cultures. The phenol degradation V^* spread over 9 to 450 mg O₂ g DW⁻¹ h⁻¹ in the range of 5 to 410 mg L⁻¹ phenol (S^*).

The TCE transformation kinetics exhibited both Michaelis-Menton and Haldane type of kinetic curves. The transformation capacities and yields were within a smaller range, but several strains with high T_C and T_y were identified.

PG02, type III and V, PG01 and type II were considered as suitable candidates for efficient TCE co-metabolism application.

7.1.6 Designing, construction and operation of a phenol-TCE alternative degradation reactor

A glass reactor was designed to facilitate alternative phenol and TCE degradation by phenol-grown aerobic granules. The reactors were inoculated with phenol-grown aerobic granules cultured in larger 2 L reactors, and TCE-phenol alternatively fed test was conducted for 6 weeks.

The phenol degradation in all three reactors (two TCE, one control) was found to be complete, and the phenol in the influent was degraded within 30 min.

The two TCE-fed reactors also showed good TCE degradation activity, and were able to completely remove the fed TCE within the cycle time limit. The reactor achieved 90~95% TCE removal efficiency across the entire test duration.

The granules in the TCE reactors exhibited lower SOUR and SVI value, and higher SS and size than that in the control reactor. The granules in the TCE reactors were also able to fully retain their structural integrity, round shape, and good settling ability after 6 weeks of operation. However, granules in the control reactor almost totally disintegrated and nearly no granular structure was observed.

7.2 Recommendations on further study

Some of the findings made in this research were interesting, e.g. the unique response of aerobic granules to TCE transformation product toxicity. However, due to the limited time and resources, they were not further explored. Therefore, it is intended in this section to point out some promising directions for the future research on the TCE co-metabolism by phenol-grown aerobic granules, or other related areas.

The recommended future research can be conducted on:

7.2.1 Using different medium, loading and aeration rate to cultivate granules

The aerobic granules obtained in this research were cultivated with established methods, i.e. methods that had been proven by previous studies to be able to produce stable granules. The purpose of it was to obtain mature granules for further study in a short time. However, it is not known how the granules would behave in TCE co-metabolism if they were cultivated under different conditions. The conditions may include, but not limited to the medium composition and operational parameters.

The most important factor in the medium composition that is likely to influence TCE degradation by the cultivated granules may be the carbon-nitrogen ratio. Researches have found that when the growth substrate is in excess for the maximum growth rate (unbalanced growth), many organisms can synthesize storage polymers (Nichols & White, 1989). The excess substrate can be converted to the polymers and accumulated within the cells as storage granules, e.g. poly- β -hydroxybutyrate (PHB) granules. It was also suggested that the carbon-nitrogen ratio in the feeding had effect on the cellular PHB level, as methanotrophs produced more PHB when grown at high methane-nitrogen ratio (Asenjo and Suk, 1986).

TCE degradation under aerobic conditions requires reducing energy in the cells. The reducing energy could be provided by the degradation of the substrate, an exogenous

electron donor, or the endogenous electron source. The latter includes the storage polymers, e.g. PHB. When the cells are grown under sequencing batch mode, as is the case in aerobic granule cultivation, feast and famine periods succeed each other. As the concentration of the substrate during the famine period is nearly zero, the reducing power supply might be limited. Therefore, if the granular sludge has high storage of PHB, the TCE degradation during the famine period may be more sustainable.

A higher carbon-nitrogen ratio in the feeding may increase the accumulation of the storage granules, and provide for reducing energy during the famine period of each cycle. The microscopic observation of the aerobic granules for the PHB substance is also recommended as a confirmation.

7.2.2 Other operational parameters likely to affect the granules' performance in TCE degradation

- Aeration rate

The aeration rate in the granular sludge reactor directly affects the structure and strength of the granules, hence the diffusion limitation, and even microbial diversity within the granules.

- Phenol loading rate

Phenol loading rate can be adjusted by changing phenol concentration in the influent, the cycle time and the volumetric exchange ratio. Each parameter may cause difference in the granule's physiological and genetic characteristics.

7.2.3 The cause of the unique response to TCE toxicity, the possible protection mechanism and its application

The most extraordinary phenomenon observed in this study is the unique TCE transformation product toxicity effect on the granules. The granules seemed to be highly robust to the toxicity caused by TCE transformation. Smaller dosage of TCE could increase the subsequent phenol-dependent SOUR. Even when the transformation capacity

was exhausted, the granules were able to immediately resume phenol degradation with considerable rate. The reason for such a phenomenon was tentatively explored, and it was proposed that the reactive species were detoxified within the granules (Chapter Four). However, due to the limited time and methods, the mechanism of such a protection against TCE toxicity, especially the enhancing effect of small TCE dosages was not further explored. It will be highly interesting for scientific research and relevant to industrial application to further study this phenomenon in the future work.

7.2.4 Various TCE and phenol loading, and operational conditions for the glass reactor

The small, glass reactors for TCE and phenol alternative degradation were operated for 6 weeks, under fixed TCE and phenol loading, as the aim of the operation was to see if the design and operation scheme were feasible. As the aim was realized, and the granules showed considerable TCE degradation activity and structural stability, it might be advisable to experiment different TCE loading, phenol loading, cycle time, recirculation rate etc. in the next step, to fully explore the ability of aerobic granules in TCE co-metabolism application. Such information will be useful in further scaling up of this process.

7.2.5 Shift of microbial diversity during TCE degradation, and its relationship to the kinetic behaviors and physiological properties of the individual isolates

In the operation of TCE-phenol alternative degradation reactors (Chapter Six), only parameters regarding the general characteristics of the granules (i.e. size, SVI, SOUR) were collected. The change of the bacteria in the granules on the population level was not quantified. However, it is feasible and interesting to know what effect TCE degradation operation has on the bacterial population shifting within the granules.

The isolation of pure phenol-degrading bacteria from the granules was conducted in Chapter Five, and the isolates were characterized for their phenol and TCE kinetics and other physiological properties. As stated in the conclusions of Chapter Five, some isolates

seemed to be more suitable in the TCE co-metabolism application, because of their high degradation rate, transformation capacity or growth yield. Therefore it could be expected that these bacteria will have a selective advantage in the mixed population of the granules, when experiencing repetitive TCE and phenol degradation. Compared to the bacteria that have low TCE transformation capacity, (and hence higher sensitivity to the toxicity), or low growth yield on phenol, the bacteria with the advantages will be less stressed by TCE co-metabolism, and grow faster, causing the shifting in the relative bacterial abundance, and also their spacial distribution in the granules.

On the other hand, the shifting of bacterial abundance will also cause the change of TCE and phenol degradation activity exhibited by the whole granules, and the change of granular sludge characteristics. In order to further understand the interaction of TCE degradation and bacterial ecology in the granule, it would be advisable to monitor the bacterial population shift during TCE degradation operation by advanced methods, e.g. competitive PCR and denaturing gradient gel electrophoresis (DGGE). Such knowledge will also be useful in the further application of aerobic granules in co-metabolism application.

REFERENCES

- Aiba S., Shoda M. & Nagalani M. **1968** Kinetics of product inhibition in alcohol fermentation. *Biotechnol. Bioeng.* **10**: 845~864
- Anderson JF. & McCarty PL. **1994** Model for treatment of trichloroethylene by methanotrophic biofilms. *Journal of Environmental Engineering* **120(2)**: 379~399
- Alexander M. **1981** Biodegradation of chemicals of environmental concern. *Science* **211**: 132~138
- Alexander M. **1999** Biodegradation and bioremediation (2nd ed.). Academic Press, San Diego, US., 1~16, 249~268
- Allsopp D., Seal K. & Gaylarde C. **2004** Introduction to biodeterioration. Cambridge University Press, UK., 1~10
- Alvarez-Cohen L. & McCarty PL. **1991a** A cometabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity. *Environ. Sci. Technol.* **25(8)**: 1381~1386
- Alvarez-Cohen L. & McCarty PL. **1991b** Product toxicity and cometabolic competitive inhibition modeling of chloroform and trichloroethylene transformation by Methanotrophic resting cells. *Appl. Environ. Microbiol.* **57(4)**: 1031~1037
- Alvarez-Cohen L. & McCarty PL. **1991c** Effect of toxicity, aeration and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. *Appl. Environ. Microbiol.* **57(1)**: 228~235
- Alvarez-Cohen L. & McCarty PL. **1991d** Two-stage dispersed growth treatment of halogenated aliphatic compounds by cometabolism. *Environ. Sci. Technol.* **25(8)**: 1387~1392
- APHA **1998** Standard methods for the examination of water and waster water. 20th edn. American Public Health Association, Washington, D.C
- Arp DJ. **1995** Understanding the diversity of trichloroethene co-oxidations. *Current Opinion in Biotechnology* **6**: 352~358

- Arvin E. **1991** Biodegradation kinetics of chlorinated aliphatic hydrocarbons with methane oxidizing bacteria in an aerobic fixed biofilm reactor. *Wat. Res.* **25(7)**: 873~881
- Asenjo JA. & Suk JS. **1986** Microbial conversion of methane into poly- β -hydroxybutyrate (PHB): growth and intercellular product accumulation in a type II methanotroph. *J. Ferment. Technol.* **64**: 271~278
- Ayala-del-Río L. **2002** Long-term effects of phenol and phenol plus trichloroethene application on microbial communities in aerobic sequencing batch reactors. Ph.D. dissertation, Michigan State University, East Lansing, Michigan, US.
- Ayoubi PJ. & Harker AR. **1998** Whole-cell kinetics of trichloroethylene degradation by phenol hydroxylase in a *Ralstonia eutropha* JMP134 derivative. *Appl. Environ. Microbiol.* **64(11)**: 4353~4356
- Bailey JE. & Ollis DF. **1986** The kinetics of enzyme-catalyzed reactions. In: Carberry JJ. et al. (ed.) *Biochemical engineering fundamentals*. (pp.86~156) McGraw Hill, Singapore
- Barnett M. **1990** Microbiology laboratory exercises (2nd ed.) Wm. C. Brown Publishers, 192~193
- Benson HJ. **2002** Microbiological applications (laboratory manual in general microbiology) (8th ed.) McGraw Hill, 160
- Bergmann JG. & Sanik J. **1957** Determination of trace amounts of chlorine in naphtha. *Anal. Chem.* **29**: 241~243
- Beun JJ., Hendriks A., van Loosdrecht MCM., Morgenroth E., Wilderer PA. & Heijnen JJ. **1999** Aerobic granulation in a sequencing batch reactor. *Wat. Res.* **33(10)**: 2283~2290
- Beun JJ., van Loosdrecht MCM. & Heijnen JJ. **2000** Aerobic granulation. *Water Science and Technology* **41(4~5)**: 41~48
- Beun JJ. **2001** PHB metabolism and N-removal in sequencing batch granular sludge reactors. Ph. D dissertation, Delft Univ Technol, Netherlands
- Beun JJ., van Loosdrecht MCM. & Heijnen JJ. **2002** Aerobic granulation in a sequencing batch airlift reactor. *Wat. Res.* **36**: 702~712

- Boopathy R. & Peters R. **2001** Enhanced biotransformation of trichloroethylene under mixed electron acceptor conditions. *Current Microbiology* **42**: 134~138
- Bradley PM. & Chapelle FH. **2000** Aerobic microbial mineralization of dichloroethene as sole carbon source. *Environ. Sci. Technol.* **34**: 221~223
- Broholm K., Jensen BK., Christensen TH. & Olsen L. **1990** Toxicity of 1,1,1-trichloroethane and trichloroethene on a mixed culture of methane-oxidizing bacteria. *Appl. Environ. Microbiol.* **56**: 2488~2493
- de Bruin WP., Kotterman MJJ., Posthumus MA., Schraa G. & Zehnder AJB **1992** Complete biological reductive transformation of tetrachloroethylene to ethane. *Appl. Environ. Microbiol.* **58**: 1996~2000
- Chang HL. & Alvarez-Cohen L. **1995a** Model for the cometabolic biodegradation of chlorinated organics. *Environ. Sci. Technol.* **29**: 2357~2367
- Chang HL. & Alvarez-Cohen L. **1995b** Transformation capacities of chlorinated organics by mixed cultures enriched on methane, propane, toluene, or phenol. *Biotechnol. Bioeng.* **45**: 440~449
- Chu KH. & Alvarez-Cohen L. **1996** Trichloroethylene degradation by methane-oxidizing cultures grown with various nitrogen sources. *Water Environment Research* **68(1)**: 76~82
- Chu KH. & Alvarez-Cohen L. **1999** Evaluation of toxic effects of aeration and trichloroethylene oxidation on methanotrophic bacteria grown with different nitrogen sources. *Appl. Environ. Microbiol.* **65(2)**: 766~772
- Cobb GD. & Bouwer EJ. **1991** Effects of electron acceptors on halogenated organic compound biotransformations in a biofilm column. *Environ. Sci. Technol.* **25**: 1088~1074
- Cohen DB., Bowes GW. **1984** Water quality and pesticides: a California risk assessment program. California State Water Resources Control Board, Sacramento
- Colby J., Stirling DI. & Dalton H. **1977** The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath). *Biochem. J.* **165**: 395~402
- Coyle GC., Parkin GF. & Gibson DT. **1993** Aerobic phenol-induced TCE degradation in completely mixed continuous-culture reactors. *Biodegradation* **4**: 59~69

- Dabrock B., Riedel J., Bertram J. & Gottschalk G. **1992** Isopropylbenzene (cumene) –a new substrate for the isolation of trichloroethylene-degrading bacteria. *Arch. Microbiol.* **158**: 9~13
- Dagley S. **1985** Microbial metabolism of aromatic compounds. In: Moo-Young M (ed) *Comprehensive biotechnology*. Pergamon, Oxford New York Toronto, pp 483~505
- Dalton H., Stirling. DI. **1982** Co-metabolism. *Philos. Trans. R. Soc. Lond. B* **297**: 481~496
- Dalton H., Prior SD , Leak DJ. & Stanley SH. **1984** Regulation and control of methane monooxygenase. P. 75~82. In: Crowford RL. & Hanson RS. (ed.) *Microbial growth on C₁ compounds*. Proceedings of the 4th International Symposium American Society for Microbiology, Washington DC.
- Davidson PM. & Branen AL. **1981** Antimicrobial activity of non-halogenated phenolic compounds. *J. Food Prot.* **44**: 623~632
- Dilling WL., Tefertiller NB., Kallos GJ. **1975** *Environ. Sci. Technol.* **9**: 833~838
- DiSpirito AA., Gullledge J., Shiemke AK., Murrell JC., Lidstrom ME. & Krema CL. **1992** Trichloroethylene oxidation by the membrane-associated methane monooxygenase in type I, type II and type X methanotrophs. *Biodegradation* **2**: 151~164
- DiStefano TD., Gossett JM. & Zinder SH. **1991** Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. *Appl. Environ. Microbiol.* **57(8)**: 2287~2292
- Doi Y. **1990** *Microbial polyesters*. New York: VCN Publishers, 156
- Dolan ME. & McCarty ML. **1995** Methanotrophic chloroethene transformation capacities and 1,1-dichloroethene transformation product toxicity. *Environ. Sci. Technol.* **29**: 2741~2747
- Dolfing J., van de Wijngaard, Janssen DB. **1993** Microbiological aspects of the removal of chlorinated hydrocarbons from air. *Biodegradation* **4**: 261~282
- Dombek PE., Johnson LK., Zimmerley ST. & Sadowsky MJ. **2000** Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. *Appl. Environ. Microbiol.* **66(6)**: 2572~2577
- Edwards VH. **1970** The influence of high substrate concentration on microbial kinetics. *Biotechnol. Bioeng.* **12**: 679~712

- Ely RL., Williamson KJ., Guenther RB., Hyman MR. & Arp DJ. **1995** A cometabolic kinetics model incorporating enzyme inhibition, inactivation, and recovery. *Biotechnology and Bioengineering* **46**: 218~245
- Ensign SA., Hyman MR. & Arp DJ. **1992** Cometabolic degradation of chlorinated alkenes by alkene monooxygenase in a propylene-grown *Xanthobacter* strain. *Appl. Environ. Microbiol.* **58**(9): 3038~3046
- Ensley, BD. **1991** Biochemical diversity of trichloroethylene metabolism. *Annu. Rev. Microbiol.* **45**: 283~299
- Ensley BD. & Kurisko PR. **1994** A Gas Lift Bioreactor for Removal of Contaminants from the Vapor-Phase. *Appl. Environ. Microbiol.* **60**(1): 285~290
- Etterer T. & Wilderer PA. **2001** Generation and properties of aerobic granular sludge. *Water Science and Technology* **43**(3): 19~26
- Ewers JD., Freier-Schröder & Knackmuss HJ. **1990** Selection of trichloroethylene (TCE) degrading bacteria that resist inactivation by TCE. *Arch. Microbiol.* **154**: 410~413
- Fan SF. & Scow KM **1993** Biodegradation of trichloroethylene and toluene indigenous microbial populations in soil. *Appl. Environ. Microbiol.* **59**(6): 1911~1918
- Fantroussi SE., Naveau H. & Agathos S N. **1998** Anaerobic dechlorinating bacteria. *Biotechnol. Prog.* **14**: 167~188
- Fitch MW., Speitel GE. & Georgiou G. **1993** Degradation of trichloroethylene by methane-grown cultures of *Methylosinus trichosporium* PP358. *Appl. Environ. Microbiol.* **62**(3): 1124~1128
- Fitch MW., Weissman D., Phelps P., Georgiou G. & Speitel G. Jr **1996** Trichloroethylene degradation by *Methylosinus trichosporium* OB3b mutants in a sequencing biofilm reactor. *Wat. Res.* **30**(11): 2655~2664
- Flemming HC. & Wingender J. **2001** Relevance of microbial extracellular polymeric substances (EPSs). *Water Science and Technology* **43**(6): 1~16
- Folsom BR., Chapman PJ. & Pritchard PH., **1990** Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: Kinetics and interactions between substrates. *Appl. Environ. Microbiol.* **56**(5): 1279~1285

- Folsom BR. & Chapman PJ. **1991** Performance characterization of a model bioreactor for the biodegradation of trichloroethylene by *Pseudomonas cepacia* G4. Appl. Environ. Microbiol. **57(6)**: 1602~1608
- Fox BG., Borneman JG., Wackett LP., Lipscomb JD. **1990** Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: Mechanistic and environmental implications. Biochemistry **29**: 6419~6427
- Freedman DL. & Gossett JM **1989** Biological reductive dechlorination of tetrachloroethylen and trichloroethylene to ethylene under methanogenic conditions. Appl. Environ. Microbiol. **55**: 2144~2151
- Fries MR., Forney LJ. & Tiedje JM. **1997** Phenol- and toluene-degrading microbial populations from an aquifer in which successful trichloroethene cometabolism occurred. Appl. Environ. Microbiol. **63(4)**: 1523~1530
- Fujita M., Ike M., Hioki JI., Kataoka K. & Takeo M. **1995** Trichloroethylene degradation by genetically engineered bacteria carrying cloned phenol catabolic genes. Journal of Fermentation and Bioengineering **79(2)**: 100~106
- Futamata H., Harayama S. & Watanabe K. **2001a** Diversity in kinetics of trichloroethylene-degrading activities exhibited by phenol-degrading bacteria. Appl. Microbiol. Biotechnol. **55**: 248~253
- Futamata H., Harayama S. & Watanabe K. **2001b** Group-specific monitoring of phenol hydroxylase genes for a functional assessment of phenol-stimulated trichloroethylene bioremediation. Appl. Environ. Microbiol. **67(10)**: 4671~4677
- Futamata H., Harayama S., Hiraishi A. & Watanabe K. **2003** Functional and structural analyses of trichloroethylene-degrading bacterial communities under different phenol-feeding conditions: laboratory experiments. Appl. Microbiol. Biotechnol. **60**: 594~600
- Gossett JM. **1987** Measurement of Henry's law constants for C1 and C2 chlorinated hydrocarbons. Environ. Sci. Technol. **21**: 202~208
- Green DW. **1984** Perry's chemical engineers' handbook, 6th ed., p. 12~45. McGraw-Hill, Inc., NewYork, N.Y.
- Guiot S., Pauss A. & Costerton J. **1992** A structured model of the anaerobic granule consortium. Wat. Sci. Tech. **25(7)**: 1~10

- Hamamura N., Page C., Long T., Semprini L. & Arp DJ. **1997** Chloroform cometabolism by butane-grown CF8, *Pseudomonas butanovora*, and *Mycobacterium vaccae* JOB5 and methane-grown *Mythosinus trichosporium* OB3b. Appl. Environ. Microbiol. **63(9)**: 3607~3613
- Han K. & Levenspiel O. **1988** Extended mono kinetics for substrate, product and cell inhibition. Biotechnol. Bioeng. **32**: 430~437
- Hanada S. et al. **1998** Phylogenetic analysis of trichloroethylene-degrading bacteria newly isolated from soil polluted with this contaminant. Journal of Fermentation and Bioengineering **86(6)**: 539~544
- Hao OJ., Kim MH., Seagren EA. & Kim H. **2002** Kinetics of phenol and chlorophenol utilization by Acinetobacter species. Chemosphere **46**: 797-807
- Harker AR. & Kim Y. **1990** Trichloroethylene degradation by two independent aromatic-degrading pathways in *Alcaligenes eutrophus* JMP134. Appl. Environ. Microbiol. **56(4)**: 1179~1181
- Hata J., Miyata N., Kim ES., Takamizawa K. & Iwahori K. **2004** Anaerobic degradation of *cis*-1,2-dichloroethylene and vinyl chloride by *Clostridium* sp. Strain DC1 isolated from landfill leachate sediment. J. Bioscience and Bioengineering **97(3)**: 196~201
- Heald S. & Jenkins RO. **1994** Trichloroethylene removal and oxidation toxicity mediated by toluene dioxygenase of *Pseudomonas putida*. Appl. Environ. Microbiol. **60(12)**: 4634~4637
- Hecht V., Brebbermann D., Bremer P. & Deckwer W-D. **1995** Cometabolic degradation of trichloroethylene in a bubble column bioscrubber. Biotechnol. Bioengineering **47**: 46~469
- Henry SM & Grbić-Galić D. **1991a** Influence of endogenous and exogenous electron donor and trichloroethylene oxidation toxicity on trichloroethylene oxidation by methanotrophic cultures from a groundwater aquifer. Appl. Environ. Microbiol. **55**: 236~244
- Henry SM & Grbić-Galić D. **1991b** Inhibition of trichloroethylene oxidation by the transformation intermediate carbon monoxide. Appl. Environ. Microbiol. **57(6)**: 1770~1776

- Henrysson T. & McCarty PL. **1993** Influence of the endogenous storage lipid poly- β -hydroxybutyrate on the reducing power availability during cometabolism on trichloroethylene and naphthalene by resting methanotrophic mixed cultures. Appl. Environ. Microbiol. **59(5)**: 1602~1606
- Henschler D., Hoos WR., Fetz H., Dallmeier E. & Metzler M. **1979** Reactions of trichloroethylene epoxide in aqueous systems. Biochem. Pharmacol. **28**: 543~548
- Hino S., Watanabe K. and Takahashi N. **1998** Phenol hydroxylase cloned from *Ralstonia eutropha* strain E2 exhibits novel kinetic properties. Microbiology **144**: 1765-1772
- Holliger C., Wohlfarth G. & Diekert G. **1999** Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol. Reviews **22**: 383~398
- Hopkins GD., Munakata J., Semprini L. & McCarty PL. **1993a** Trichloroethylene concentration effects on pilot field-scale *in-situ* groundwater bioremediation by phenol-oxidizing microorganisms. Environ. Sci. Technol. **27**: 2542~2547
- Hopkins GD. Semprini L. & McCarty PL. **1993b** Microcosm and in situ field studies of enhanced biotransformation of trichloroethylene by phenol-utilizing microorganisms. Appl. Environ. Microbiol. **59(7)**: 2277~2285
- Hopkins GD. & McCarty PL. **1995** Field evaluation of in situ aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as the primary substrates. Environ. Sci. Technol. **29**: 1628~1637
- Horvath RS. **1972** Microbial cometabolism and the degradation of organic compounds in nature. Bacteriol. Rev. **36**: 146~155
- Hou CT. **1984** Appl. Microbiol. Biotechnol. **19**: 1~4
- van Hylckama Vlieg JET., de Koning W. & Janssen DB. **1996** Transformation kinetics of chlorinated ethenes by *Methylosinus trichosporium* OB3b and detection of instable epoxides by on-line gas chromatography. Appl. Environ. Microbiol. **62(9)**: 3304~3312
- van Hylckama Vlieg JET., de Koning W. & Janssen DB. **1997** Effect of chlorinated ethene conversion on viability and activity of *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **63(12)**: 4961~4964
- Hyman MR., Russell SA., Ely RL., Williamson KJ. & Arp DJ. **1995** Inhibition, inactivation, and recovery of ammonia-oxidizing activity in cometabolism of

- trichloroethylene by *Nitrosomonas europaea*. Appl. Environ. Microbiol. **61**(4): 1480~1487
- Janssen DB. & Witholt B. **1992** Aerobic and anaerobic degradation of haologated aliphatics. In: Sigel H. & Sigel A. (ed.) Metal Ions in Biological Systems, Vol 28, Degradation of Environmental Pollutants by Microorganisms and Their Metalloenzyems (pp 299~327), Marcel Dekker, New York
- Jiang HL., Tay JH. & Tay STL. **2002** Aggregation of immobilized activated sludge cells into aerobically grown microbial granules for the aerobic biodegradation of phenol. Letters in Applied Microbiology **35**: 1~7
- Jiang HL., Tay JH. & Tay STL. **2004a** Changes in structure, activity and metabolism of aerobic granules as a microbial response to high phenol loading. Appl. Microbiol. Biotechnol. **63**:602~608
- Jiang HL., Tay JH., Maszenan AM. & Tay ST-L. **2004b** Bacterial diversity and function of aerobic granules engineered in a sequencing batch reactor for phenol degradation. Appl. Environ. Microbiol. **70**(11): 6767~6775
- Keener WK. & Arp DJ. **1993** Kinetic studies of ammonia monooxygenase inhibition in *Nitrosomonas europaea* by hydrocarbons and halogenated hydrocarbons in an optimized whole-cell assay. Appl. Environ. Microbiol. **59**(8): 2501~2510
- Keweloh H. Heipieper HJ. Rehm HJ. **1989** Protection of bacteria against toxicity of phenol by immobilization in calcium alginate. Appl. Microbiol. Biotechnol. **31**: 383~389
- Keweloh H., Weyrauch G. & Rehm H-J. **1990** Phenol-induced membrane changes in free and immobilized *Escherichia coli*. Appl. Microbiol. Biotechnol. **53**: 66~71
- Krumme ML., Timmis KN. & Dwyer DF. **1993** Degradation of trichloroethylene by *Pseudomonas cepacia* G4 and the constitutive mutant strain G4 5223 PR1 in aquifer microcosms. Appl. Environ. Microbiol. **59**(8): 2746~2749
- Landa AS., Sipkema EM., Weijma J., Beenackers AACM., Dolfing J. & Janssen DB. **1994** Cometabolic degradation of trichloroethylene by *Pseudomonas cepacia* G4 in a chemostat with toluene as the primary substrate. Appl. Environ. Microbiol. **60**(9): 3368~3374

- Leahy JG., Byrne AM. & Olsen RH. **1996** Comparison of factors influencing trichloroethylene degradation by toluene-oxidizing bacteria. Appl. Environ. Microbiol. **62(3)**: 825~833
- Li SY. & Wackett LP. **1992** Trichloroethylene oxidation by toluene dioxygenase. Biochem. Biophysical Res. Commun. **185(1)**: 443~451
- Li Y. & Liu Y. **2005** Diffusion of substrate and oxygen in aerobic granule. Biochemical Engineering Journal **27**: (45~52)
- Liu Y., Yang SF., Liu QS. & Tay JH. **2003** The role of cell hydrophobicity in the formation of aerobic granules. Current Microbiol. **46(4)**: 270~274
- Liu YQ., Liu Y. & Tay, JH. **2004a** The effect of extracellular polymeric substances on the formation and stability of biogranules. Appl. Microbiol. Biotechnol. **65**: 143~148
- Liu Y., Yang SF., Li Y., Xu H., Qin L. & Tay, JH. **2004b** The influence of cell and substratum surface hydrophobicities on microbial attachment. J. Biotechnol. **110**: 251~256
- Liu YQ., Tay Jh., Ivanov V., Moy BY-P., Liu Y. & Tay ST-L. **2005a** Influence of phenol on nitrification by microbial granules. Process Biochemistry **40**: 3285~3289
- Liu YQ., Liu Y. & Tay JH. **2005b** Relationship between size and mass transfer resistance in aerobic granules. Letters in Appl. Microbiol. **40**: 312~315
- Liu Y., Wang ZW., Qin L., Liu YQ. & Tay JH. **2005c** Selection pressure-driven aerobic granulation in a sequencing batch reactor. Appl. Microbiol. Biotechnol. **67(1)**: 26~32
- Loh KC., Chung TS. & Ang WF. **2000** Immobilized-cell membrane bioreactor for high strength phenol wastewater. J. Environ. Engineering **126**: 75~79
- Lowe M., Madson EL., Schindler K., Smith C., Emrich S., Robb F. & Halden RU. **2002** Geochemistry and microbial diversity of a trichloroethene-contaminated superfund site undergoing intrinsic in situ reductive dechlorination. FEMS Microbiology Ecology **40**: 1213~134
- Mägli A., Wendt M. & Leisinger T. **1996** Isolation and characterization of *Dehalobacterium formicoaceticum* gen. nov. sp. nov., a strictly anaerobic bacterium utilizing dichloromethane as source of carbon and energy. Arch. Microbiol. **16**: 101~108

- Malachowsky KJ., Phelps TJ., Teboli AB., Minnikin DE. & White DC. **1994** Aerobic mineralization of trichloroethylene, vinyl chloride, and aromatic compounds by *Rhodococcus* species. Appl. Environ. Microbiol. **60**(2): 542~548
- Maltoni C & Gurengerich FP. **1974** Carcinogenicity bioassays of vinyl chloride: Research plan and early results. Environ. Res. **7**: 387~396
- Mars AE., Houwing J., Dolfing J. & Janssen DB. **1996** Degradation of toluene and trichloroethylene by *Burkholderia cepacia* G4 in growth-limited fed-batch culture. Appl. Environ. Microbiol. **62**(3): 886~891
- Mars AE., Prins GT., Wietzes P., de Koning W. & Janssen DB. **1998** Effect of trichloroethylene on the competitive behavior of toluene-degrading bacteria. Appl. Environ. Microbiol. **64**(1): 208~215
- McCarty P. **1997** Breathing with chlorinated solvents. Science **276**: 1521~1522
- McClay K., Streger SH. & Steffan RJ. **1995** Induction of toluene oxidation activity in *Pseudomonas mendocina* KR1 and *Pseudomonas* sp. strain ENVPC5 by chlorinated solvents and alkanes. Appl. Environ. Microbiol. **61**: 3479~3481
- McFarland MJ., Vogel CM. & Spain JC. **1992** Methanotrophic cometabolism of trichloroethylene (TCE) in a two stage bioreactor system. Water Research **26**: 259~265
- McSwain BS., Irvine RL., Hausner M. & Wilderer PA. **2005** Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. Appl. Environ. Microbiol. **71**(2): 1051~1057
- The Merck Index: an encyclopedia of chemicals, drugs and biologicals. **2001** O'Neil, Maryadele J. Merck & Co. Whitehouse Station, NJ : Merck, 2001.
- Miller RE. & Guengrich FP. **1983** Metabolism of trichloroethylene in isolated hepatocytes, microsomes and reconstituted enzyme systems containing cytochrome P-450. Cancer Res. **43**: 1145~1152
- Monoro Y., Unno H., Tanji Y. & Hori K. **2004** Addition of aromatic substrates restores trichloroethylene degradation activity in *Pseudomonas putida* F1. Appl. Environ. Microbiol. **70**(5): 2830~2835
- Morgenroth E., Sherden T., van Loosdrecht MCM., Heijnen JJ. & Wilderer **1997** Aerobic granular sludge in a sequencing batch reactor. Water Research **31**(12): 3191~3194

- Moy BYP., Tay JH., Toh SK., Liu Y. & Tay STL. **2002** High organic loading influences the physical characteristics of aerobic sludge granules. *Letters in Applied Microbiology* **34**: 407~412
- Mu DY. & Scow KM. **1994** Effect of trichloroethylene (TCE) and toluene concentrations on TCE and toluene biodegradation and the population density of TCE and toluene degraders in soil. *Appl. Environ. Microbiol.* **60**(7): 2661~2665
- Nelson MJK., Montgomery SO. & Pritchard PH. **1988** Trichloroethylene metabolism by microorganisms that degrade aromatic compounds. *Appl. Environ. Microbiol.* **54**: 604~006
- Newman LM. & Wackett LP. **1995** Purification and characteriazation of toluene 2-monooxygenase from *Burkholderia cepacia* G4. *Biochemistry* **34**: 14066~14076
- Newman LM. & Wackett LP. **1997** Trichloroethylene oxidation by purified toluene 2-monooxygenase: products, kinetics and turnover-dependent inactivation. *J. Bacteriol.* **179**(1): 90~96
- Nichols PD. & White DC. **1989** Accumulation of poly- β -hydroxybutyrate in a methane-enriched, halogenated hydrocarbon-degrading soil column: implications for microbial community structure and nutritional status. *Hydrobiologia* **176/177**: 369~377
- Oldenhuis RJ., Vink RLJM., Janssen DB. & Witholt B. **1989** Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. *Appl. Environ. Microbiol.* **55**: 2819~2826
- Oldenhuis RJ., Oedzes JY., van de Waarde JJ. & Janssen DB. **1991** Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. *Appl. Environ. Microbiol.* **57**(1): 7~14
- Olsen RH., Kukor JJ. & Kaphammer B. **1994** A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. *J. Bacteriol.* **176**(12): 3749~3756
- Ong SK. & Bowers AR. **1990** Treatment of inhibitory substrates. *Journal of Environmental Engineering* **116**(6): 1013-1028
- Onysko KA., Bodman HM. & Robinson CW. **2000** Effect of temperature on the inhibition kinetics of phenol biodegradation by *Pseudomonas putida* Q5. *Biotechnol. Bioengineering* **70**(3): 291~299

- Park S., Hanna ML., Taylor RT. & Droege MW **1991** Batch cultivation of *Methylosinus trichlosporium* OB3b, I: production of soluble methane monooxygenase. Biotechnol. Bioeng. **38**: 423~433
- Park J., Kukor JJ. & Abriola LM. **2002** Characterization of the adaptive response to trichloroethylene-mediated stresses in *Ralstonia pickettii* PKO1. Appl. Environ. Microbiol. **68(11)**: 5231~5240
- Patel RN., Hou CT., Laskin AI. & Felix A. **1982** Microbial oxidation of hydrocarbons: properties of a soluble methane monooxygenase from a facultative methane-utilizing organism, *Methylobacterium* sp. strain CRL-26. Appl. Environ. Microbiol. **44**: 1130~1137
- Phelps LL., Niedzielski JJ., Schram RM., Herbes SE. & White DC. **1990** Biodegradation of trichloroethylene in continuous-recycle expanded-bed bioreactors. Appl. Environ. Microbiol. **56(6)**: 1702~1709
- Qin L., Liu Y. & Tay JH. **2005** Denitrification on poly- β -hydroxybutyrate in microbial granular sludge sequencing batch reactor. Water Research **39**: 1503~1510
- Providenti MA., Lee H. & Trevors JT. **1993** Selected factors limiting the microbial degradation of recalcitrant compounds. J. Ind. Microbiol. **12**: 379~395
- Radway JC., Santo Domingo JW., Hazen TC. & Wilde EW. **1998** Evaluation of biodegradation potential of foam embedded *Burkholderia cepacia* G4. Biotechnology Letters **20(7)**: 663~666
- Rasche ME., Hyman MR. & Arp DJ. **1991** Factors limiting aliphatic chlorocarbon degradation by *Nitrosomonas europaea*: Cometabolic inactivation of ammonia monooxygenase and substrate specificity. App. Environ. Microbiol. **57(10)**: 2986~2994
- Reij MW., Kieboom J., de Bont JAM. & Hartsman S. **1995** Continuous degradation of trichloroethylene by *Xanthobacter* sp. strain Py2 during growth on propene. Appl. Environ. Microbiol. **61(8)**: 2936~2942
- Rittmann BE., Seagren E., Wrenn BA., Valocchi AJ., Ray C. & Raskin L. **1994** In situ bioremediation (2nd ed.), 1~20. Noyes Publications, Park Ridge, New Jersey, USA.
- Rittmann BE., Schwarz AO. & Saez PB. **2000** Biofilms applied to hazardous waste treatment. Biofilms: process analysis and applications, J.D. Bryers, eds., Wiley, New York, 207~234

- Ryoo D., Shim H., Canada K., Barbieri P. & Wood TK. **2000** Aerobic degradation of tetrachloroethylene by toluene-o-xylene monooxygenase of *Pseudomonas stutzeri* OX1. *Nature Biotechnol.* **18**: 775~778
- Schmidt JE. & Ahring BK. **1994** Extracellular polymers in granular sludge from different upflow anaerobic sludge blanket (UASB) reactors. *Appl. Microbiol. Biotechnol.* **42**: 457~462
- Segar RL. Jr., De Wys SL. & Speitel GE. Jr. **1995** Sustained trichloroethylene cometabolism by phenol-degrading bacteria in sequencing biofilm reactors. *Water Environment Research* **67(5)**: 764~774
- Semprini L. **1997** Strategies for the aerobic co-metabolism of chlorinated solvents. *Current Opinion in Biotechnology* **8**: 296~308
- Sheehan D. (ed.) **1997** Bioremediation protocols. Humana Press, NJ., US., 2~22
- Shields MS., Montgomery SO., Cuskey SM. Chapman PJ. & Pritchard PH. **1991** Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. *Appl. Environ. Microbiol.* **57(7)**: 1935~1941
- Shields MS. & Reagin MJ., **1992** Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. *Appl. Environ. Microbiol.* **58(12)**: 3977~3983
- Shih CC., Davey ME., Zhou JZ., Tiedje JM. & Criddle CS. **1996** Effects of phenol feeding pattern on microbial community structure and cometabolism of trichloroethylene. *Appl. Environ. Microbiol.* **62(8)**: 2953~2960
- Shingler V., Franklin F., Tsuda CH., Holroyd M. & Bagdasarian M. **1989** Molecular analysis of a plasmid-encoded phenol hydroxylase from *Pseudomonas* CF600. *J. Gen. Microbiol.* **135**: 1083~1092
- Shingleton JT., Applegate BM., Nagel AC., Bienkowske PR. & Sayler GS. **1998** Induction of the *tod* operon by trichloroethylene in *Pseudomonas putida* TVA8. *Appl. Environ. Microbiol.* **64(12)**: 5049~5052
- Shurtliff MM., Parkin GF., Weathers LJ. & Gibson DT. **1996** Biotransformation of trichloroethylene by a phenol-induced mixed culture. *J. Environ. Engineering* **122(7)**: 581~589
- Sikkema J., de Bont JAM. & Poolman B. **1995** Mechanism of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **59**:201~222

- Simon BH., Bader J., Günther H., Neumann S. & Thanos J. **1985** Chiral compounds synthesized by biocatalytic reductions. *Angew. Chem. Int. Ed. Engl.* **24**: 539~553
- Singh A. & Ward OP. **2004** Biodegradation and bioremediation. Springer, **1~17**
- Sokol W. & Howell JA. **1981** Kinetics of phenol oxidation by washed cells. *Biotechnol. Bioeng.* **23**: 2039~2049
- Spain JC., Zylstra GJ., Blake CK. & Gibson DT. **1989** Monohydroxylation of phenol and 2,5-dichlorophenol by toluene dioxygenase in *Pseudomonas putida* F1. *Appl. Environ. Microbiol.* **55**: 2648~2652
- Strand SE., Wodrich JV. & Stensel HD. **1991** Biodegradation of chlorinated solvents in a sparged, methanotrophic biofilm reactor. *Research Journal WPCF* **63(6)**: 859~867
- Strandberg GW., Donaldson TL. & Farr LL. **1989** Degradation of trichloroethylene and *trans*-1,2-dichloroethylene by a methanotrophic consortium in a fixed-film, packed-bed bioreactor. *Environ. Sci. Technol.* **23**: 1422~1425
- Storck W. **1987** Chlorinated solvent use hurt by federal rules. *Chem. Eng. News* **65**: 11
- Sun AK. & Wood TK. **1996** Trichloroethylene degradation and mineralization by pseudomonads and *Methylosinus trichosporium* OB3b. *Appl. Microbiol. Biotechnol.* **45**: 248~256
- Sun AK. & Wood TK. **1997** Trichloroethylene mineralization in a fixed-film bioreactor using a pure culture expressing constitutively toluene *ortho*-monooxygenase. *Biotechnol. Bioeng.* **55(4)**: 674~685
- Tally WJ. (ed.) **2006** Bioremediation of recalcitrant compounds. Taylor & Francis Group, CRC press, **1~9**
- Tay JH., Liu QS. & Liu Y. **2001a** The effect of shear force on the formation, structure and metabolism of aerobic granules. *Appl. Microbiol. Biotechnol.* **57**: 227~233
- Tay JH., Liu QS. & Liu Y. **2001b** The role of cellular polysaccharides in the formation and stability of aerobic granules. *Letters in Applied Microbiology* **33**: 222~226
- Tay JH., Liu QS. & Liu Y. **2002a** Characteristics of aerobic granules grown on glucose and acetate in sequential aerobic sludge blanket reactor. *Environ. Technol.* **23**: 931~936
- Tay JH., Ivanov V., Pan S. & Tay ST-L. **2002b** Specific layers in aerobically grown microbial granules. *Letters in Applied Microbiology* **34**:1~4

- Tay JH., Yang SF. & Liu Y. **2002c** Hydraulic selection pressure-induced nitrifying granulation in sequencing batch reactors. *Appl. Microbiol. Biotechnol.* **59**: 332~337
- Tay JH., Liu QS. & Liu Y. **2004a** The effect of upflow air velocity on the structure of aerobic granules cultivated in a sequencing batch reactor. *Water Science and Technology* **49(11-12)**: 35~40
- Tay JH., Jiang HL., Tay ST-L. **2004b** High-rate biodegradation of phenol by aerobically grown microbial granules. *Journal of Environm. Engineering-Asce* **130(12)**: 1415~1423
- Tschantz MF., Bowman JP., Donaldson TL. Bienkowski PR. et al. **1995** Methanotrophic TCE biodegradation in a multi-stage bioreactor. *Environ. Sci. Technol.* **29**: 2073~2082
- Tsien HC., Brusseau GA., Hanson RS. & Wackett LP. **1989** Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. *Appl. Environ. Microbiol.* **55**: 3155~3161
- Vannelli T., Logan M., Arciero DM. & Hooper AB. **1990** Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Appl. Environ. Microbiol.* **56(4)**: 1169~1171
- Verce MF., Ulrich RL. & Freedman DL. **2000** Characterization of and isolate that uses vinyl chloride as a growth substrate under aerobic conditions. *Appl. Environ. Microbiol.* **66(8)**: 3535~3542
- Villaverde S. & Fernandez-Polanco F. **1999** Spatial distribution of respiratory activity in *Pseudomonas putida* 54G biofilms degrading volatile organic compounds (VOC) *Appl. Microbiol. Biotechnol.* **51**: 382~387
- Vogel TM & McCarty P **1985** Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride and carbon dioxide under methanogenic conditions. *Appl. Environ. Microbiol.* **49**: 1080~1083
- Vogel TM., Criddle CS., McCarty PL. **1987** Transformations of halogenated aliphatic compounds: *Environ. Sci. Technol.* **21**: 722~736
- Wackett LP. & Householder SR. **1989** Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenase. *Appl. Environ. Microbiol.* **55**: 2723~2725
- Wang ZH., Liu Y. & Tay JH. **2006** The role of SBR mixed liquor volume exchange ratio in aerobic granulation. *Chemosphere* **62**: 767~771

- Ward OP., Singh A. & Van Hamme J. **2003** Accelerated biodegradation of petroleum hydrocarbon waste. J. Ind. Microbiol. Biotechnol. **30**: 260~270
- Watanabe K., Hino S., & Takahashi N. **1996a** Responses of activated sludge to an increase in phenol loading. Journal of Fermentation and Bioengineering **82(5)**: 522~524
- Watanabe K., Hino S., Onodera K., Kajie SI. & Takahashi N. **1996b** Diversity in kinetics of bacterial phenol-oxygenating activity. J. Ferment. Bioeng. **81(6)**: 560~563
- Wayman M. & Tseng MC. **1976** Inhibition threshold substrate concentrations. Biotechnol. Bioeng. **18**: 383~387
- Webb JL. **1963** Enzyme and metabolic inhibitor. Boston: Academic Press
- Westrick JJ. **1990** National surveys of volatile organic compounds in ground and surface waters. In Significance and treatment of volatile organic chemicals in water supplies. Lewis Publishers, Inc., Ann Arbor, Mich., 103
- Whited GM. & Gibson DT. **1991** Separation and partial characterization of the enzyme of the toluene-4-monooxygenase catabolic pathway in *Pseudomonas mendocina* KR1. J. Bacteriol. **173(9)**: 3017~3020
- van den Wijngaard AJ., Wind RD. & Hanssen DB. **1993** Kinetics of bacterial growth on chlorinated aliphatic compounds. Appl. Environ. Microbiol. **59**: 2041~2048
- Wilcox DW., Autenrieth RL. & Bonner JS. **1994** Propane-induced biodegradation of vapor phase trichloroethylene. Biotechnol. Bioengineering **46**: 333~342
- Wilén BM., Gapes D. & Keller J. **2004a** Determination of external and internal mass transfer limitation in nitrifying microbial aggregates. Biotechnol. Bioengineering **86(4)**: 446~457
- Wilén BM., Gapes D., Blackall LL. & Keller J. **2004b** Structure and microbial composition of nitrifying microbial aggregates and their relation to internal mass transfer effects. Water Research **50(10)**: 213~220
- Wilson JT., Wilson BH. **1985** Biotransformation of trichloroethylene in soil. Appl. Environ. Microbiol. **49**: 242~243
- Winter RB., Yen KM., Ensley BD. **1989** Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. BioTechnology **7**: 282~285
- Yang RD. & Humphrey AE. **1975** Dynamic and steady-state studies of phenol biodegradation in pure and mixed cultures. Biotechnol. Bioeng. **17**: 1211~1235

- Yano T., Nakahara T., Kamiyama S. & Yamada K. **1966** Kinetic studies on microbial activities in concentrated solutions. I. Effect of excess sugars on oxygen uptake rate of a cell-free respiratory system. *Agr. Biol. Chem.* **30**: 42~48
- Yap LF., Lee YK. & Poh CL. **1999** Mechanism for phenol tolerance in phenol-degrading *Comamonas testosteroni* strain. *Appl. Microbiol. Biotechnol.* **51**: 833~840
- Yaskovich GA. **1998** The role of cell surface hydrophobicity in adsorption immobilization of bacteria strains. *Appl. Biochem. Microbiol.* **34**: 373~376
- Yeager CM., Bottomley PJ. & Arp DJ. **2001a** Requirement of DNA repair mechanisms for survival of *Burkholderia cepacia* G4 upon degradation of trichloroethylene. *Appl. Environ. Microbiol.* **67**: 5364~5391
- Yeager CM., Bottomley PJ. & Arp DJ. **2001b** Cytotoxicity associated with trichloroethylene oxidation in *Burkholderia cepacia* G4. *Appl. Environ. Microbiol.* **67**(5): 2107~2115

Publications:

- Co-metabolism of Trichloroethylene by Phenol-Grown Aerobic granules: Formation of Aerobic Granules and Kinetic Studies, submitted to Water Research.
- TCE Co-metabolism by Phenol Grown Aerobic Granules: Rate Limiting Factors and Toxicity Study, submitted to Biotechnology and Bioengineering.
- An Aerobic Granular Sludge Blanket Reactor for Sustainable, Alternative Trichloroethylene and Phenol Degradation, in preparation.