Chapter 5

Microbial Degradation of Persistent Organophosphorus Flame Retardants

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Flame retardants

Flame retardants (FRs) are chemicals used in polymers to protect the public from accidental fires by preventing or retarding the initial phase of a developing fire (EFRA, 2007). These chemicals are now found in numerous consumer products, including construction materials, upholstery, carpets, electronic goods, furniture and also children's products such as car seats, strollers and baby clothing. FRs have become indispensable to modern life, and have saved numerous lives by preventing unexpected fires across the globe.

FRs are divided into two general classes based on their relation to host polymers: additive and reactive FRs (WHO, 1997). Additive FRs are simply mixed with host polymers. The lack of chemical bonding between the FRs and host polymers enables the FRs to leach out of or volatilize from host polymers over time into the ambient environment. Reactive FRs are incorporated into host polymers by covalent bonding into the polymer backbone, and are thus less likely to leach into the environment. Additive FRs are mainly used in thermoplastics, textiles and rubbers, whereas reactive FRs are usually used in thermoset plastics and resins (SFT, 2009a).

FRs are sub-divided into six groups characterized by their chemical composition: 1) aluminum hydroxide, 2) brominated, 3) organophosphorus, 4) antimony oxides, 5) chlorinated and 6) other FRs. These groups account for 40%, 23%, 11%, 8%, 7% and 11% of the annual FR global consumption in 2007, respectively (Beard & Reilly, 2009). The total market for FRs in the United States, Europe and Asia in 2007 amounted to about 1.8 million tons.

1.2. Organophosphorus flame retardants

Organophosphorus flame retardants (PFRs) are based primarily on phosphate esters, phosphonate esters and phosphite esters. The total consumption of FRs in Europe was an estimated 465,000 tons in 2006, of which 20% comprised PFRs (KLIF, 2010). Of the PFRs consumed, 55% were chlorinated. Halogenated PFRs are the preferred form of FRs because halogen inhibits flame formation in organic materials, and non-halogenated PFRs are typically used as flame-retardant plasticizers (KLIF, 2010).

1.3. Tris(1,3-dichloro-2-propyl) phosphate and tris(2-chloroethyl) phosphate

Tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and tris(2-chloroethyl) phosphate (TCEP) are typical examples of additive chlorinated PFR (Fig. 1 and Table 1).

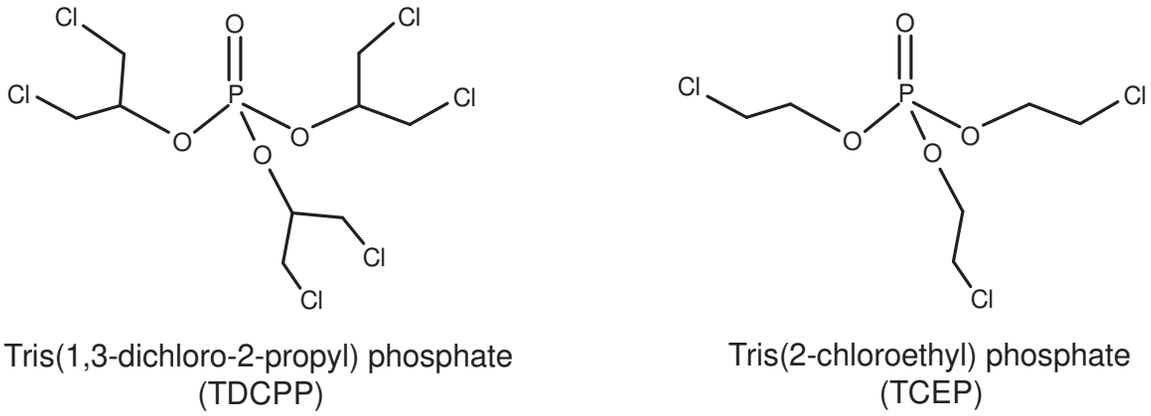

Figure 1. Chemical structure of tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and tris(2-chloroethyl) phosphate (TCEP)

TDCPP is a viscous colorless to light yellow liquid and is produced by the epoxide opening of epichlorohydrin in the presence of phosphorus oxychlorine (ATSDR, 2009). TDCPP is used primarily in flexible polyurethane foams but also in rigid polyurethane foams, resins, plastics, textile coatings and rubbers (California EPA, 2011). TDCPP was a common ingredient of sleepwear for children in the 1970s, but was voluntarily withdrawn by manufactures in 1977 because of its proven mutagenicity (California EPA, 2011). However, the PFR can still be found in many baby products (Stapleton et al., 2011). Currently, TDCPP is used mostly in flexible polyurethane foams for upholstered furniture and automotive products. TDCPP consumption has increased following the ban on common FR polybrominated diphenyl ethers (PBDEs). Consequently, total TDCCP production has increased, being an estimated 4,500-22,700 tons in the United States in 2006 and <10,000 tons in Europe in 2000 (van der Veen & de Boer, 2012).

TCEP is colorless to pale yellow liquid and is highly soluble in water (Fig. 1 and Table 1). The compound is chemically synthesized via condensation of phosphorus oxychloride and chloroalkyl alcohol at low temperatures and pressures to avoid formation of alkyl chlorides (ATSDR, 2009). Previously, the main purpose of TCEP was to reduce the

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brittleness of flame-resistant rigid or semirigid polyurethane foams. More recently, it has been used as a flame-retarding plasticizer and viscosity regulator in unsaturated polyester resin (accounting for around 80% of current use) (EURAR, 2009). TCEP-containing polymers are commonly used in the furniture, textile and building industries (for example, more than 80% of the TCEP consumption in the EU is invested in roofing insulation). TCEP is also used in car, railway and aircraft materials, and in professional paints. Since the 1980s, TCEP has been progressively replaced by other flame retardants, primarily tris(1-chloro-2-propyl) phosphate (TCPP). Consequently, global consumption of TCEP in the EU, which exceeded 9,000 tons in 1989, declined to below 4,000 tons by 1997. TCEP is no longer produced in the EU (EURAR, 2009).

	tris(1,3-dichloro-2-propyl) phosphate (US EPA, 2005)	tris(2-chloropropyl) phosphate (EURAR, 2009)
Cas number:	13674-87-8	115-96-8
Synonym:	Tris(1,3-dichloro-2-propyl) phosphate Tris-(2-chloro-,1-chloromethyl-ethyl)-phosphate 1,3-dichloro-2-propanol phosphate Phosphoricacid, tris(1,3-dichloro-2-propylester) Tris(1,3-dichloroisopropyl) phosphate Tris(1-chloromethyl-2-chloroethyl) phosphate Tri(β, β'-dichloroisopropyl) phosphate	Tris(2-chloroethyl) phosphate Tris(β-chloroethyl) phosphate 2-chloroethanol phosphate Phosphoricacid,tris(2-chloroethyl) ester Tris(2-chloroethyl) orthophosphate Tris(chloroethyl) phosphate
Abbreviation:	TDCPP TDCP	TCEP TClEP
Molecular weight:	430.91	285.49
Physical state:	Viscous, clear liquid	Clear, transparent, Low viscosity liquid
Melting point:	-58°C	<-70°C
Boiling point:	236-237°C at 5 mm Hg	Decomposition at 320°C at 1013 hPa
Density:	1.52	1.4193 (25°C)
Vapor pressure:	0.01 mmHg (30°C)	43 Pa (136.9°C) 0.00114 Pa (20°C, extrapolated)
Water solubility:	42 mg/L	7.82 g/L (20°C)
n-Octanol/water partition coefficient:	2.4	1.78

Table 1. General aspect of Tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and tris(2-chloroethyl) phosphate (TCEP)

1.4. Occurrence and behavior of TDCPP and TCEP in the environment

TCEP and TDCPP have been detected in various environments worldwide, including indoor and outdoor air, surface and ground waters, and even drinking water (Tables 2 and 3). It is unlikely that these compounds are produced naturally. Their environmental presence is thus considered to be the result of human activity. Because these PFRs are physicochemically and microbiologically stable in the environment and are also reportedly toxic, they are a serious threat to human and ecosystem health.

1.4.1. TDCPP

Detected air concentrations of TDCPP have attained up to150 ng m^{3-1} in Sweden houses, and in Belgium office and stores, they have reached 73 ng m^{3-1} (Table 2). In outdoor air, TDCPP levels near a main road in Sweden ranged from <0.04-0.072 m^{3-1}, and significant amounts have been detected globally in air borne particles over the Pacific, Indian, Arctic and Southern Oceans. TDCPP has been also found in indoor dust at relatively higher concentrations. Levels of TDCPP have tended to be higher in public buildings than in domestic buildings.

With respect to water environments, TDCPP concentrations have been detected at up to ~50 ng L^{-1} in German rivers and at 1,335 ng L^{-1} in Italian lakes. In these countries, it also occurs in rain and/or snow, as a result of volatilization from host materials. A much higher TDCPP concentration was detected in raw water at a disposal site in Japan, suggesting that the compound leaches and migrates to water sources. In the United States and Germany, TDCPP has even been detected in drinking water processed in treatment plants (DWTs). Relatively higher concentrations of TDCPP occur in landfill site sediments. Much higher concentrations still have been found in sediments near a car demolition site in Norway.

TDCPP has been also detected in the effluents of sewage treatment plants (STPs) and waste water treatment plants (WWTPs) in European countries and Japan, revealing that effluents are a source of aquatic TDCPP contamination. Comparable levels have been observed in the influents, indicating that the compound persists in the treatment plants. Degradation of TDCPP in the environment has been reported as low. Together, these observations suggest that TDCPP is likely to accumulate in the environment.

Environment	Concentration	Location	Country	Reference
Indoor air:	<0.04-18 ng m^{3-1}	office and store	Norway	SFT, 2008
	<0.2-150 ng m^{3-1}	home, cinema, university, hospital, hotel, prison, library, office shops	Sweden	Marklund et al., 2005a
	<0.3-7 ng m^{3-1}	lecture and computer hall, electronic dismantling facility recycling plant	Sweden	Staaf & Ostman, 2005
	<73 ng m^{3-1}	work place	Belgium	Bergh et al., 2011
	<61.4 ng m^{3-1}	house	Japan	Kanazawa et al., 2010

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Environment	Concentration	Location	Country	Reference
	1.3 ng m^{3-1}	newly constructed house	Japan	Saito et al., 2007
	<0.6 ng m^{3-1}, <8.7 ng m^{3-1}	house and office	Japan	Saito et al., 2007
Indoor dust:	0.2-67 μg g^{-1}	home, cinema, university, hospital, hotel, prison, library, office shops	Sweden	Marklund et al., 2003
	<0.08-6.64 μg g^{-1}	house	Belgium	van den Eede et al., 2011
	<0.08-56.2 μg g^{-1}	store	Belgium	van den Eede et al., 2011
	2.2-27 μg g^{-1}	home	Belgium	Bergh et al., 2011
	3.9-150 μg g^{-1}	day care	Belgium	Bergh et al., 2011
	3.3-91 μg g^{-1}	work place	Belgium	Bergh et al., 2011
	<1.1 μg g^{-1}	house	Spain	Garcia et al., 2007
	<0.09-56.1 μg g^{-1}	house	United States	Stapleton et al., 2009
	0.069-18 μg g^{-1}	hotel	Japan	Takigami et al., 2009
	<127 μg kg^{-1}	house	Japan	Kanazawa et al., 2010
Outdoor air:	<0.04-0.072 ng m^{3-1}	nearby main road	Sweden	Marklund et al., 2003
	<0.04-0.14 ng m^{3-1}	remote area from main road	Sweden	Marklund et al., 2003
	n.d.-5 pg m^{3-1}	sea	Arctic ocean	Moller et al., 2012
	16-52 pg m^{3-1}	sea	Japan	Moller et al., 2012
	5-8 pg m^{3-1}	sea	Northern pacific ocean	Moller et al., 2012
	49-780 pg m^{3-13}	sea	East Indian archipelago, Philippine sea	Moller et al., 2012
	n.d.-220 pg m^{3-13}	sea	Indian ocean	Moller et al., 2012
	80 pg m^{3-1}	sea	Southern ocean	Moller et al., 2012
Surface water:	10-18 ng L^{-1}	river	Germany	Andresen & Bester, 2006
	~50 ng L^{-1}	river	Germany	Andresen et al., 2004
	2-24 ng L^{-1}	rain	Germany	Regnery & Püttmann, 2009
	5-40 ng L^{-1}	snow	Germany	Regnery & Püttmann, 2009
	<19 ng L^{-1}	river	Austria	Martinez-Carballo et al., 2007
	<3.0-19 ng L^{-1}	river	Austria	Martinez-Carballo et al., 2007
	<1,335 ng L^{-1}	lake	Italy	Bacaloni et al., 2008
	108-448 ng L^{-1}	rain	Italy	Bacaloni et al., 2008
	680-6,180 ng L^{-1}	raw water of waste disposal site	Japan	Kawagoshi et al., 1999
Drinking water:	1.2-2.4 ng L^{-1}	water after drinking water treatment	Germany	Andresen & Bester, 2006
	<250 ng L^{-1}	water after drinking water treatment	United States	Stackelberg et al., 2004

Environment	Concentration	Location	Country	Reference
Sediment:	<0.15-54 μg kg^{-1}	lake and fjord at vicinity of WWFP	Norway	KLIF, 2010
	1,500-4,100 μg kg^{-1}	landfill site	Norway	SFT, 2008
	<250-8,800 μg kg^{-1}	car demolition site	Norway	SFT, 2008
	<709 μg kg^{-1}	waste disposal site	Japan	Kawagoshi et al., 1999
Sludge:	110-330 μg kg^{-1}		Norway	SFT, 2008
	3.0-260 μg kg^{-1}		Sweden	Stackelberg et al., 2004
Influent:	630-820 ng L^{-1}	WWTP	Norway	SFT, 2008
	240-450 ng L^{-1}	STP	Sweden	Marklund et al., 2005b
	330-1,600 ng L^{-1}	STP	Japan	Ishikawa et al., 1985
Effluent:	86-740 ng L^{-1}	WWTP	Norway	SFT, 2008
	130-340 ng L^{-1}	STP	Sweden	Marklund et al., 2005b
	20-120 ng L^{-1}	STP	Germany	Andresen et al., 2004
	19-1,400 ng L^{-1}	WWTP	Austria	Martinez-Carballo et al., 2007
	280-1,400 ng L^{-1}	STP	Japan	Ishikawa et al., 1985
Biota:	<6.0 ng g^{-1}	fish liver	Norway	SFT, 2009b
	<0.3-6.7 ng g^{-1}	fish muscle	Norway	SFT, 2009b
	<0.72-1.9 ng g^{-1}	bird egg	Norway	KLIF, 2010
	<0.11-0.16 ng g^{-1}	bird blood and plasma	Norway	KLIF, 2010
	<0.6-8.1 ng g^{-1}	whole fish	Norway	SFT, 2009b
	<1.5 ng g^{-1}	seabird liver	Norway	SFT, 2009b
	<0.3-1.2 ng g^{-1}	whole fish liver	Norway	SFT, 2009b
	<5.0 ng g^{-1}, <10-<30 ng g^{-1}	cod liver and mussel	Norway	SFT, 2008
	49-140 ng g^{-1}	freshwater fishes close to sources	Norway	Sundkvist et al., 2010
	16.-5.3 ng g^{-1}	human milk	Sweden	Sundkvist et al., 2010

Table 2. Occurrence and behavior of TDCPP

TDCPP has also been detected in biological samples, including fishes, mussels and birds. In Norway, fishes and mussels were observed to contain up to 8.1 and 30 ng g^{-1} of TDCPP, respectively. In bird blood/plasma and eggs respectively, TDCPP levels range from <0.11-0.16 and from <0.72-1.9 ng g^{-1}. In Sweden, freshwater fishes close to emission sources contained 49-140 ng g^{-1} TDCPP. Worryingly, TDCPP has also been detected in the breast milk of Swedish women.

1.4.2. TCEP

In Sweden, the highest detected air concentration of TCEP was 730 ng m^{3-1} inside an office furnished with linoleum floor and a new photocopier (Table 3). In outdoor air, it can reach 6.2 ng m^{3-1} beside a main road, but remote areas harbor less than 0.2 ng m^{3-1}, implicating

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road traffic as an important source of TCEP emission. TCEP has also been detected globally in air borne particles over the Pacific, Indian, Arctic and Southern Ocean. In Belgium, indoor dust can contain up to 260 μg g^{-1} TCEP. TCEP concentrations in dusts of public spaces tend to exceed those in domestic dusts.

TCEP ranges from <3.0-1,236 ng L^{-1} in German rivers, lakes and reservoirs. In this country and in Italy, it has also been detected in rain and/or snow, indicating that, like TDCPP, TCEP volatilizes from its host materials. Groundwater TCEP levels up to 754 ng L^{-1} have been reported in Germany, suggesting that TCEP primarily mobilizes into water rather than attaching to soil. TCEP also occurs in drinking water or finished water from DWTs; recorded concentrations are as high as 99, 25 and 1.7 ng L^{-1} in the United States, Korea and Germany, respectively. Much higher concentrations have been observed in raw water of waste disposal sites in Japan. Relatively higher concentrations of TCEP have been detected in landfill site sediments in Japan and Norway (up to 7,400 and 380 μg kg^{-1}, respectively). Especially high concentrations were found in the sediment nearby a car demolition site.

TCEP has been also detected in STP or WWTP effluents in many countries. Comparable levels of TCEP are observed in the influents. These observations demonstrate that, like TDCPP, TCEP persists in the treatment plants.

Also similarly to TDCPP, TCEP has been detected in biological samples, including fishes, crabs, mussels and birds. In Norway, fishes and mussels respectively contain up to 26 and 23 ng g^{-1} TCEP. In birds and their eggs, TCEP levels can reach up to 6.1 ng g^{-1}. In fishes residing near emission sources in Sweden, they reach up to 69 and 160 ng g^{-1} respectively. Furthermore, like TDCPP, TCEP has been detected in the breast milk of Swedish women.

Environment	Concentration	Location	Country	Reference
Indoor air:	<0.2-23 ng m^{3-1}	office and store	Norway	SFT, 2008
	3, 9 ng m^{3-1}	lecture room and kindergarten	Sweden	Tollback et al., 2006
	0.4-730 ng m^{3-1}	home, cinema, university, hospital, hotel, prison, library, office shops	Sweden	Marklund et al., 2005a
	<0.3-10 ng m^{3-1}	Lecture and computer hall, electronic dismantling facility recycling plant	Sweden	Staaf & Ostman, 2005
	<22 ng m^{3-1}	car, theater, furniture store, office and electronics store	Sweden	Hartmann et al., 2004
	3-15 ng m^{3-1}	lecture room and office room	Sweden	Bjorklund et al., 2004
	<297 ng m^{3-1}	house	Japan	Kanazawa et al., 2010
	<136 ng m^{3-1}, <42.1 ng m^{3-1}	house and office	Japan	Saito et al., 2007
	1.2 ng m^{3-1}	newly constructed house	Japan	Saito et al., 2007
	<28 ng m^{3-1}	home	Belgium	Bergh et al., 2011
	7.8-230 ng m^{3-1}	day care center	Belgium	Bergh et al., 2011

Environment	Concentration	Location	Country	Reference
	<140 ng m^{3-1}	work place	Belgium	Bergh et al., 2011
Indoor dust:	0.19-94 µg g^{-1}	home, cinema, university, hospital, hotel, prison, library, office shops	Sweden	Marklund et al., 2003
	<0.08-2.65 µg g^{-1}	house	Belgium	van den Eede et al., 2011
	<33 µg g^{-1}	house	Belgium	Bergh et al., 2011
	<0.08-5.46 µg g^{-1}	store	Belgium	van den Eede et al., 2011
	2.5-150 µg g^{-1}	day care center	Belgium	Bergh et al., 2011
	1.3-260 µg g^{-1}	work place	Belgium	Bergh et al., 2011
	0.25-1.56 µg g^{-1}	house	Spain	Garcia et al., 2007
	<308 µg g^{-1}	house	Japan	Kanazawa et al., 2010
	0.082-2.3 µg g^{-1}	hotel	Japan	Takigami et al., 2009
Outdoor air:	0.51-6.2 ng m^{3-1}	nearby main road	Sweden	Marklund et al., 2003
	<0.2 ng m^{3-1}	remote area from main road	Sweden	Marklund et al., 2003
	126-585 pg m^{3-1}	ocean	Arctic ocean	Moller et al., 2012
	273-1,961 pg m^{3-1}	sea	Japan	Moller et al., 2012
	159-282 pg m^{3-1}	sea	Northern pacific ocean	Moller et al., 2012
	19-156 pg m^{3-1}	sea	East Indian archipelago, Philippine sea	Moller et al., 2012
	46-570 pg m^{3-1}	sea	Indian ocean	Moller et al., 2012
	74 pg m^{3-1}	sea	Southern ocean	Moller et al., 2012
Surface water:	<3-184 ng L^{-1}	lake and reservoir	Germany	Regnery & Püttmann , 2010
	12-130 ng L^{-1}	river	Germany	Andresen & Bester, 2006
	13-130 ng L^{-1}	river	Germany	Andresen et al., 2004
	<1,236 ng L^{-1}	river	Germany	Fries & Püttmann , 2003
	11-196 ng L^{-1}	rain	Germany	Regnery & Püttmann, 2009
	121 ng L^{-1}	rain	Germany	Fries & Püttmann , 2003
	19-60 ng L^{-1}	snow	Germany	Regnery & Püttmann, 2009
	13-130 ng L^{-1}	river	Austria	Martinez-Carballo et al., 2007
	<33 ng L^{-1}	lakes	Italy	Bacaloni et al., 2008
	7 ng L^{-1}	river	Italy	Bacaloni et al., 2007
	19-161 ng L^{-1}	rain	Italy	Bacaloni et al., 2008
	4,230-87,400 ng L^{-1}	raw water of waste disposal site	Japan	Kawagoshi et al., 1999
	14-347 ng L^{-1}	river and sea water	Japan	Ishikawa et al., 1985
	14-81 ng L^{-1}	lake and river	Korea	Kim et al., 2007
Ground water:	3-9 ng L^{-1}		Germany	European Commission DG ENV, 2011
	<312 ng L^{-1}		Germany	Fries & Püttmann , 2003

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Environment	Concentration	Location	Country	Reference
	<754 ng L^{-1}		Germany	Fries & Püttmann , 2001
Drinking water:	0.74-1.7 ng L^{-1}	water after drinking water treatment	Germany	Andresen & Bester, 2006
	4-99 ng L^{-1}	water after drinking water treatment	United States	Stackelberg et al., 2007
	<99 ng L^{-1}	water after drinking water treatment	United States	Stackelberg et al., 2004
	14, 25 ng L^{-1}	water after drinking water treatment	Korea	Kim et al., 2007
Sediment:	<0.16-8.5 µg kg^{-1}	lake and fjord at vicinity of WWFP	Norway	KLIF, 2010
	27-380 µg kg^{-1}	landfill site	Norway	SFT, 2008
	2,300-5,500 µg kg^{-1}	car demolition site	Norway	SFT, 2008
	<160 µg kg^{-1}	river	Austria	Martinez-Carballo et al., 2007
	<7,400 µg kg^{-1}	waste disposal site	Japan	Kawagoshi et al., 1999
Sludge:	<9-<19 µg kg^{-1}		Norway	SFT, 2008
	6.6-110 µg kg^{-1}		Sweden	Marklund et al., 2005b
Influent:	2,000-2,500 ng L^{-1}	STP	Norway	SFT, 2008
	90-1,000 ng L^{-1}	STP	Sweden	Marklund et al., 2005b
	290, 180 ng L^{-1}	STP	Germany	Meyer & Bester, 2004
	983-1,123 ng L^{-1}	municipal STWs	Germany	Fries & Püttmann , 2003
	<0.025-0.3 ng L^{-1}	STP	Spain	Rodriguez et al., 2006
	540-1,200 ng L^{-1}	STP	Japan	Ishikawa et al., 1985
Effluent:	1600-2,200 ng L^{-1}	STP	Norway	SFT, 2008
	350-890 ng L^{-1}	STP	Sweden	Marklund et al., 2005b
	350, 370 ng L^{-1}	STP	Germany	Meyer & Bester, 2004
	214-557 ng L^{-1}	municipal STWs	Germany	Fries & Püttmann , 2003
	<0.025-0.7 ng L^{-1}	STP	Spain	Rodriguez et al., 2006
	500-1,200 ng L^{-1}	STP	Japan	Ishikawa et al., 1985
Biota:	0.5-5.0 ng g^{-1} 13-26 ng g^{-1}	fish muscle and liver	Norway	SFT, 2009b
	1.8-3.2 ng kg^{-1}	whole fish	Norway	SFT, 2009b
	<5 ng g^{-1}, <10-23 ng g^{-1}	cod liver and mussel	Norway	SFT, 2008
	<0.6-4.7 ng g^{-1}	sea bird liver	Norway	SFT, 2009b
	<0.17-19 ng g^{-1}	beach crab	Norway	KLIF, 2010
	<0.06-0.11 ng g^{-1}	blue mussel	Norway	KLIF, 2010
	<1.7-8.6 ng g^{-1}	burbot liver	Norway	KLIF, 2010
	<0.08-0.21 ng g^{-1}	trout	Norway	KLIF, 2010
	<0.33-6.1 ng g^{-1}	bird egg	Norway	KLIF, 2010
	<0.17-6.0 ng g^{-1}	bird blood and plasma	Norway	KLIF, 2010

Environment	Concentration	Location	Country	Reference
	1.5-69 ng g^{-1}	marine fishes	Sweden	Sundkvist et al., 2010
	<160 ng g^{-1}	freshwater fishes close to sources	Sweden	Sundkvist et al., 2010
	201-8.2 ng g^{-1}	human milk	Sweden	Sundkvist et al., 2010

Table 3. Occurrence and behavior of TCEP

1.5. Toxicological information of TDCPP and TCEP

Since the toxic effects of TCEP and TDCPP have been regarded as marginal compared to those of PBDEs, they have been extensively used. However, their non-negligible toxicities have been revealed in a number of studies (Tables 4 and 5). Together with their persistence in the environment, the environmental contamination of both compounds has become of serious concern.

1.5.1. TDCPP

Rats given oral doses of TDCPP absorb more than 90% of the compound within 24 h, with the highest concentrations being observed in kidney, liver and lung (EURAR, 2008). The acute toxicity of oral TDCPP has been reported as low, with LD_{50} values ranging from 2,250 mg kg^{-1} for female mice to 6,800 mg kg^{-1} for male rabbits (Table 4). In a 2-year chronic toxicity study in rats, the lowest observable adverse effect level (LOAEL) was 5 mg kg^{-1} day^{-1}. In that study, statistically significant relationships between TDCPP dose and tumor incidences were observed in both male and female rats. Consequently, TDCPP is today classified as Carc. Cat. 3; R40 and Cat. 2; H351, denoting "limited evidence of a carcinogenic effect" and "suspected of causing cancer", respectively.

A number of TDCPP genotoxicity studies have been conducted in whole mammals that have resulted in negative conclusions regarding genotoxicity (Albemarle Corp. & ICL North America Inc., 2011). However, *in vitro* studies using bacteria and mammalian cells have suggested that TDCPP exerts genotoxic effects, and an *in vivo* study showed its covalent binding to DNA (US EPA, 2005; Morales & Matthews, 1980).

Similarly, neurotoxicity studies of TDCPP involving hens and rats reveal no clear evidence that TDCPP is neurotoxic. However, a study based on undifferentiated and differentiating PC12 cells showed its potential neurotoxicity (Dishaw et al., 2011).

Whether, and to what extent, TDCPP is toxic to humans remains unknown. However, TDCPP has been shown to alter sex hormone balance in human cell lines, via alteration of steroidogenesis or estrogen metabolism (Liu et al., 2012). In addition, TDCPP concentrations in house dusts have been linked to altered hormone levels and decreased semen quality in men (Meeker & Stapleton, 2010).

TDCPP is regarded as toxic to aquatic organisms (EURAR, 2008). An acute toxicity study on fish trout yielded an LC_{50} value of 1.1 mg L^{-1}. Acute and chronic toxicity studies conducted on the invertebrate *Daphnia* produced an EC_{50} value of 3.8 mg L^{-1}. In a chronic study on the

alga *Pseudokirchneriella*, ErC_{10} (10% growth-rate inhibition) was recorded as 2.3 mg L^{-1}. Thus, TDCPP is classified as N; R51/53, denoting "Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment". In addition, an LC_{50} of 23 mg kg^{-1} has been reported for a terrestrial organism, the earthworm *Eisenia*.

Toxicity	Organism	Reference
Acute toxicity	LD_{50}=6,800 mg kg^{-1} male rabbit	US EPA, 2005
	LD_{50}=3,160 mg kg^{-1} male rat	EURAR, 2008
	LD_{50}=2,670 mg kg^{-1} male mice	
	LD_{50}=2,250 mg kg^{-1} female mice	
	LD_{50}=2,236 mg kg^{-1} male rat	
	LD_{50}=2,489 mg kg^{-1} female rat	
Chronic toxicity	LOAEL=5 mg kg^{-1} day^{-1} rat for hyperplasia and convoluted tubule epithelium	EURAR, 2008
Cytotoxicity	hepatocytes and neuronal cells	Crump et al., 2012
Neurotoxicity	*in vitro* PC12 cells	Dishaw et al., 2011
Carcinogenicity	rat	California EPA, 2011
Genotoxicity	*in vivo Salmonella typhimurium*	California EPA, 2011
	in vitro mouse, Chinese hamster and rat cells	
Toxic to aquatic organisms	fishes, invertebrates and algae	EURAR, 2008
	LC_{50}=1.1 mg L^{-1} rainbow trout (96 h)	
	EC_{50}=3.8 mg L^{-1} *Daphnia magana* (48 h)	
	LOEC=1.0 mg L^{-1} *Daphnia* for reproduction (21 days)	
	NOEC=0.5 mg L^{-1} *Daphnia* for reproduction (21 days)	
	ErC_{10}=2.3 mg L^{-1} algae	
	LC_{50}=23 mg kg^{-1} earthworm *Eisenia*	
	NOEC=2.9 mg kg^{-1} earthworm *Eisenia* for reproduction	
	NOEC=17 mg kg^{-1} plant *Mustard*	
Alter hormone levels	human and zebra fish cells	Liu et al., 2012
Decreased sperm quality	human	Meeker & Stapleton, 2010

Table 4. Toxicological information of TDCPP

1.5.2. TCEP

Rats given oral doses of TCEP absorb over 90% of the compound within 24 h, with marked accumulations in liver, kidney, fat and the gastrointestinal tract (EURAR, 2009). In animals,

TCEP appears to be mainly toxic to brain, kidney and liver. Toxicity studies have implicated TCEP as moderately toxic; in rats, oral administration yields an LD_{50} of 430-1,230 mg kg^{-1} and skin contact reveals a low acute dermal toxicity (LD_{50} >2,150 mg kg^{-1}) (Table 5). A 2-year chronic toxicity study of TCEP yielded LOAELs of 44 mg kg^{-1} day^{-1} in rats and 175 mg kg^{-1} day^{-1} in mice. The same study indicated that TCEP is potentially neurotoxic, with no observed adverse effect levels (NOAELs) in rats and mice being 88 mg kg^{-1} day^{-1} and 175 mg kg^{-1} day^{-1}, respectively.

Toxicity	Organism	Reference
Acute toxicity	LD_{50}=430-1,230 mg kg^{-1} rat	EURAR, 2009
	LD_{50}>2,150 mg kg^{-1} rat for dermal	EURAR, 2009
Chronic toxicity	LOAEL=44 mg kg^{-1} day^{-1} rat for kidney lesions (2 years)	EURAR, 2009
	LOAEL=175 mg kg^{-1} mouse for kidney morphology (2 years)	EURAR, 2009
Neurotoxicity	rat and mouse	EURAR, 2009
	NOAEL=88 mg kg^{-1} day^{-1} rats (16 weeks by gavage)	
	NOAEL=175 mg kg^{-1} day^{-1} mouse (16 weeks by gavage)	
Reproductive toxicity	rat and mouse	EURAR, 2009
	NOAEL=175 mg kg^{-1} day^{-1} mouse for fertility	
Carcinogenicity	rat and mouse	SCHER, 2012
Toxic to aquatic organisms	killifish, trout and goldfish	EURAR, 2009
Alter sex hormone balance	human cells and Zebra fish	Liu et al., 2012
Alter cell cycle regulatory protein expression	rabbit renal proximal tubule cells	Ren et al., 2008

Table 5. Toxicological information of TCEP

In the 2-year study, increased incidences of adenomas and carcinomas were linked to TCEP exposure, revealing TCEP as a potential carcinogen (EURAR, 2009). TCEP is thus classified as Carc. Cat. 3; R40. Because TCEP additionally exhibits reproductive toxicity in rats and mice, it is also classified as Repr. Cat. 2; R60, denoting "may impair fertility". TCEP at environmental concentrations has been reported to affect the expression of cell cycle regulatory genes in primary cultured rabbit renal proximal tubule cells (Ren et al., 2008).

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TCEP is toxic to aquatic organisms, being classified as N; R51/53 (EURAR, 2009). Short term exposure to TCEP is mildly-moderately adverse to the aquatic invertebrate organisms *Daphnia* and *Planaria,* and TCEP presents low acute toxicity to killifish, trout and goldfishes.

The toxic effects of TCEP in humans are largely unknown. However, neurotoxic signs have been reported in a 5-year old child who slept in a room with wood paneling containing 3% TCEP (Ingerowski & Ingerowski, 1997). In addition, an epidemiological study of children in school environments found a potential association between the TCEP content in air-bone dusts and impaired cognitive ability (UBA, 2008). TCEP has been further reported to alter the sex hormone balance in human cells, as well as in fish cells.

1.6. Removal technique for TDCPP and TCEP

The persistence of chlorinated FRs TCEP and TDCPP in current waste water and drinking water treatment processes has accelerated the investigation of alternative water treatment techniques that will dispel these compounds.

Echigo *et al.* showed that TDCPP in distilled water and an effluent from a solid waste landfill site is effectively degraded by O_3/vacuum UV or O_3/H_2O_2 process, although degradation products were not determined in this study (Echigo et al., 1996). Westerhoff *et al.* reported that >20% of approximately 30 ng L^{-1} of TCEP in surface water samples can be removed with powdered activated carbon, but that other adsorptive processes, metal salt coagulation and lime softening, and oxidative processes (chlorination and ozonation) are ineffective (Westerhoff et al., 2005). Lee *et al.* showed that > 90% removal efficiency of 100 μg L^{-1} of TCEP in river and sea waters is possible using tight nanofiltration membranes with a low molecular weight cutoff of approximately 200 (Lee et al., 2008). Watts *et al.* demonstrated that the higher removing efficacy (> 95%) of 5 mg L^{-1} of TCEP in a water is achieved by a UV/H_2O_2 advanced oxidation process with the highest UV fluence at 6,000 mJ cm^{-2} (Watts & Linden, 2008). In this study, the generation of stoichiometric amount of chloride ion was observed. In addition, Benotti *et al.* reported that UV/TiO_2 supplemented with H_2O_2 can decrease the concentration of TCEP in a river water, although the degradation was not so effective and not completed (Benotti et al., 2009).

2. Microbial degradation and detoxification of TDCPP and TCEP

FRs have been widely distributed commercially and are necessary to prevent or reduce mortality from accidental fires. However, the leaching of additive FRs has led to global contamination of the environment. The chlorinated PFRs TCEP and TDCPP persist in the environment and exhibit varying toxic effects, raising concerns about their effects on human and ecological health. Although several physicochemical methods for removing TCEP and TDCPP have been reported (as described above), biotechnological techniques offer an attractive alternative, being potentially cost-effective, eco-friendly and enabling *in situ* remediation of contaminants. However, prior to recent isolation of TCEP- and TDCPP-degrading bacteria by our group, no biological degrading agent for such compounds was known.

2.1. Isolation and characterization of TDCPP- and TCEP-degrading bacteria

2.1.1. Enrichment of TCEP and TDCPP-degrading bacteria

2.1.1.1. Enrichment cultivation of TCEP and TDCPP-degrading bacteria

To obtain microorganisms that can degrade TDCPP and TCEP, we used an enrichment culture technique in which one of TDCPP or TCEP served as the sole phosphorus source (Takahashi et al., 2008). Forty six environmental samples (soils and sediments) in Japan were cultivated at 30°C in minimal medium containing approximately 20 μM of each compound. Significant degradation of TCEP and TDCPP was seen in ten and three of the samples, respectively. In the first cultivation round, each compound had disappeared within 2 to 5 days; successive sub-cultivations reduced the degradation time to within one day. The enrichment cultures displaying the highest degradation efficacy against TCEP and TDCPP were designated 67E and 45D, respectively. Culture 67E completely degraded 20 μM of TDCPP in 3 h and TCEP in 6 h (Fig. 2A and B), while culture 45D completely degraded the same concentration of TDCPP in 3 h and TCEP in 24 h. During the degradations, 2-CE was liberated from TCEP and 1,3-DCP from TDCPP, indicating that the degradation pathway involved hydrolysis of phosphoester bonds.

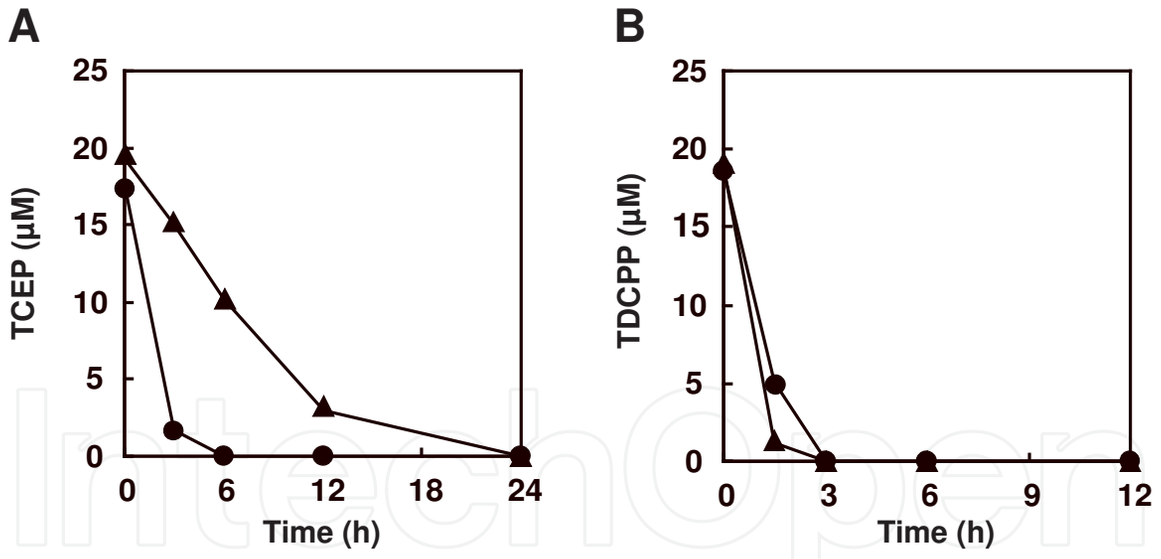

Figure 2. Degradation of TCEP (A) and TDCPP (B) by enrichment cultures. The enrichment cultures, 67E (circles) and 45D (triangles), were cultivated on 20 μM of TCEP or TDCPP as the sole phosphorus source.

2.1.1.2. 2-CE and 1,3-DCP degradation ability of enrichment cultures

The metabolites 2-CE and 1,3-DCP are also persistent and toxic: 1,3-DCP is a known genotoxin and carcinogen (NTP & NIEHS, 2005), while 2-CE exhibits genotoxicity, fetotoxicity and cardiotoxicity (National Toxicology, 1985). We analyzed whether the cultures can degrade the metabolites by measuring chloride ion formation. Cultures 67E and 45D liber-

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ated chloride ions from 2-CE and 1,3-DCP, respectively. After 120 h reaction, the proportion of chloride ion was approximately 100% and 68.5% of the total chlorine contained in the supplied 2-CE and 1,3-DCP, respectively. This shows that both cultures can dehalogenate their respective chloroalcohols and can therefore potentially detoxify chlorinated PFRs in the environment.

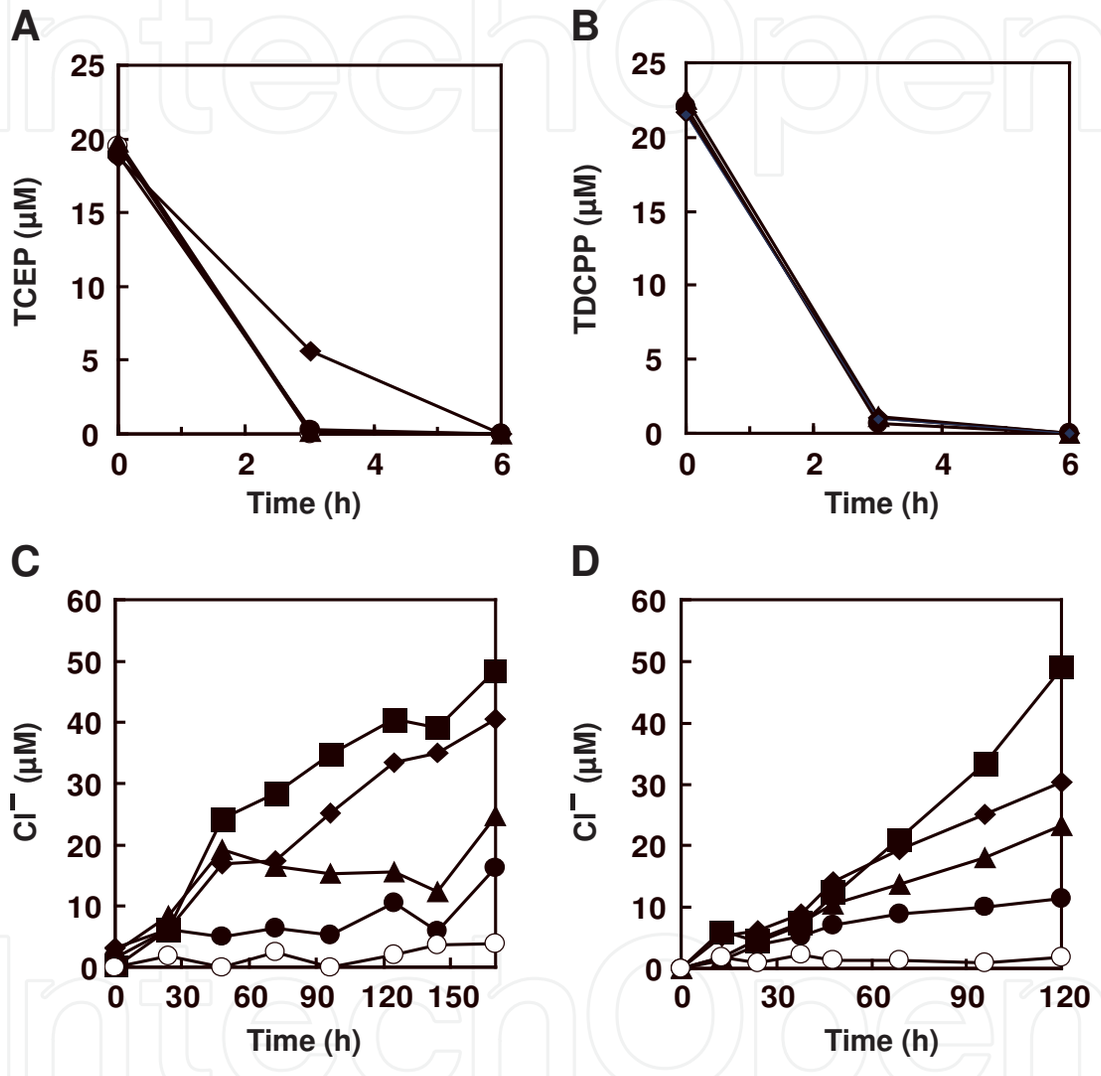

Figure 3. Effect of exogenous phosphate on the degradation of TCEP (A) and TDCPP (B) and the chloride ion formation from TCEP (C) and TDCPP (D). The enrichment cultures, 67E (A and C) and 45D (B and D), were cultivated on 20 μM of TCEP or TDCPP as the sole phosphorus source, respectively, with various concentrations of inorganic phosphate (NaH_2PO_4): 0 mM (closed circles), 0.02 mM (closed triangle), 0.2 mM (closed squares) and 2 mM (closed diamonds). Control culture without cell inoculation is indicated by open circles. Each data point represents the mean of at least two independent determinations.

2.1.1.3. Effect of exogenous phosphate on the degradation ability of enrichment cultures

Phosphate-sufficient conditions are well known to repress the expression of genes involved in phosphorus utilization. We thus examined the effect of exogenous inorganic phosphate

on TDCPP and TCEP degradations and chloride ion formation (Fig. 3). At concentrations of 0.02, 0.2 and 2 mM, exogenous inorganic phosphate did not significantly inhibit TCEP and TDCPP degradation by the respective cultures (Fig. 3A and B), but chloride ion formation was enhanced at concentrations up to 0.2 mM (Fig. 3C and D). From these results, we concluded that efficient PFR detoxification could be achieved by optimizing the inorganic phosphate concentration.

2.1.1.4. Bacterial communities of enrichment cultures

To profile the bacterial communities in the cultures, we performed denaturing gradient gel electrophoresis (DGGE) analysis (Fig. 4). In the absence of inorganic phosphate, two bands (C1 and C2) were observed in the fingerprint of TCEP-supplemented 67E, which persisted throughout cultivation (Fig. 4A). With inorganic phosphate added, the intensity of C2 markedly decreased at later incubation stages (Fig.4A). In 45D supplemented with TDCPP, a single band (D3) was observed at the beginning of cultivation, but at later times two additional bands (D1 and D2) appeared, regardless of the presence or absence of inorganic phosphate (Fig. 4B). However, with inorganic phosphate added, the intensity of D2 and D3 decreased while that of D1 increased at the late stage of cultivation (Fig. 4B). The nucleotide sequence of C1 and D1 was affiliated with the genus *Acidovorax*, that of D2 with the genus *Aquabacterium*, and C2 and D3 were assigned to the genus *Sphingomonas* (Table 6). Together with the effect of exogenous inorganic phosphate on chlorinated PFRs degradation with liberation of chloride ions, these results imply that the *Sphingomonas*-related bacteria hydrolyze the PFRs, and that the *Acidovorax*-related bacteria dehalogenate the chloroalcohols. Among these bacterial genera, a strain of S*phingomonas* sp. has been reported to hydrolyze some organophosphate pesticides, such as chlorpyrifos (Li et al., 2007). However, bacteria that are known to dehalogenate the chloroalcohols were not identified in the enrichment cultures, suggesting that a new member, possibly *Acidovorax* sp., is responsible for dehalogenating the chloroalcohols in the cultures.

Culture	Band	Phylogenetic affiliation
		Species
67E	C1	*Acidovorax* sp.
	C2	*Sphingomonas* sp.
45D	D1	*Acidovorax* sp.
	D2	*Aquabacterium* sp.
	D3	*Sphingomonas* sp.

Table 6. Phylogenetic affiliation of microorganisms represented by bands in DGGE profiles of the enrichment cultures 67E and 45D.

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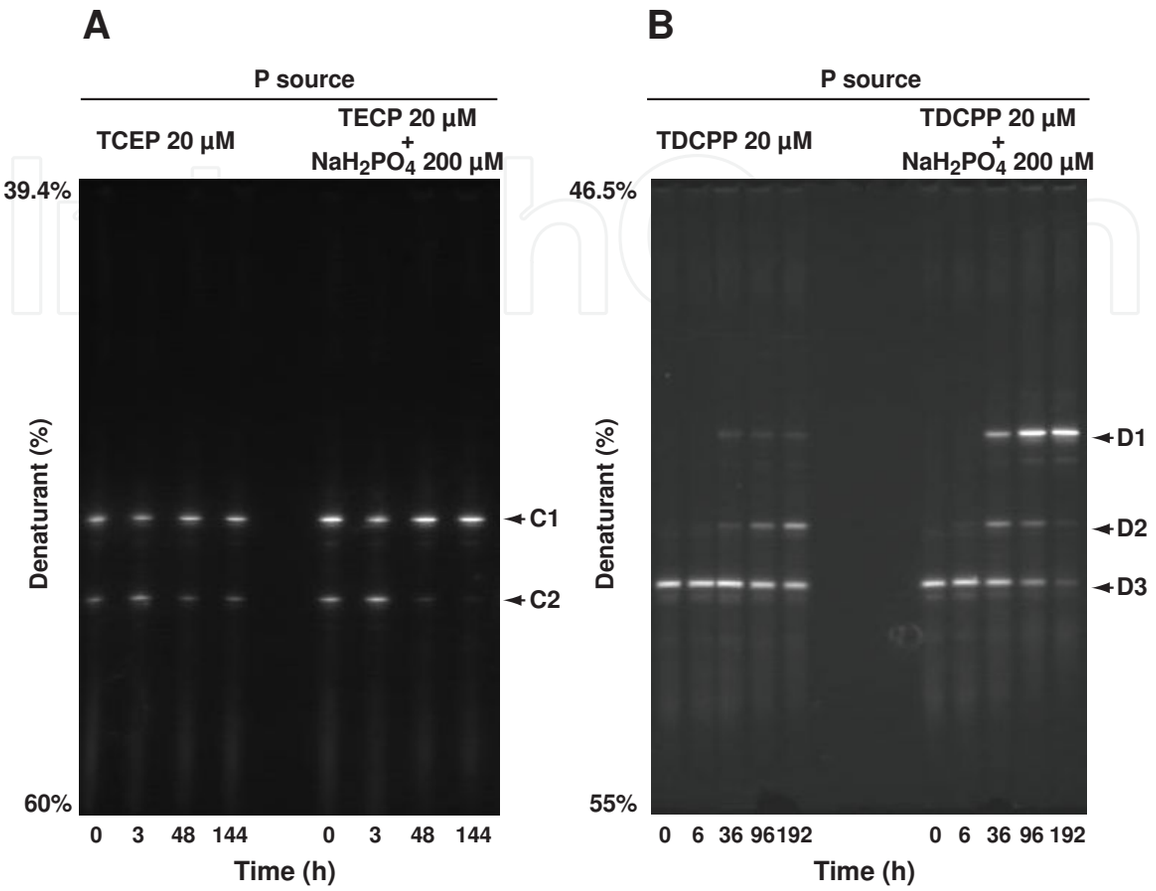

Figure 4. DGGE profile of the enrichment cultures 67E (A) and 45D (B) during cultivation in the presence of absence of inorganic phosphate. The arrowheads indicated the DNA fragments sequenced.

2.1.2. Isolation and characterization of TDCPP- and TCEP-degrading bacteria

2.1.2.1. Isolation of TDCPP- and TCEP-degrading bacteria

We attempted to isolate the bacteria responsible for degrading TDCPP and TCEP in the cultures 67E and 45D. (Takahashi et al., 2010). In the case of 45D, isolation was achieved by limiting dilution method. The culture was repeatedly serially diluted in a minimal medium containing 20 μM of TDCPP and cultivated at 30°C. Finally, the culture was spread onto a minimal agar plate containing 232 μM of TDCPP as the sole phosphorus source. A single colony grown on the plate was named strain TDK1 (Fig. 5A). In the case of 67E, the culture was spread onto a minimal agar plate containing 232 μM of TCEP as the sole phosphorus source and incubated at 30°C. Single colonies were then cultivated in a minimal medium containing 20 μM of TCEP as the sole phosphorus source. This isolation procedure was repeated three times, and a single colony was named strain TCM1 (Fig. 5B).

2.1.2.2. Identification of TDCPP- and TCEP-degrading bacteria

Both strains were short-rod-shaped bacteria (0.8-1.0 × 1.0-2.5 μm) and produced yellow, circular, convex colonies with smooth, glistening surfaces on a nutrient agar plate. As carbon sources, both strains assimilated glucose, maltose and L-arabinose; in addition, strain TCM1 assimilated potassium gluconate, while strain TDK1 assimilated D-mannose, *N*-acetyl-D-glucosamine, and D, L-malate. Both strains tested negative for indole, urease, arginine dihydrolase, nitrate reduction, gelatine hydrolysis, and glucose fermentation, and were positive for esculin hydrolysis. TCM1 and TDK1 tested negative and positive for cytochrome oxidase, respectively. The morphological and physiological characteristics of the strains were similar to those of *Sphingomonas* spp. Furthermore, the 16S rRNA gene sequence of the strains is closely related to those of sphingomonads, comprising the genera *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Takeuchi et al., 2001). The phylogenetic tree constructed from the sequences of these genera showed that strains TCM1 and TDK1 belong to *Sphingobium* and *Sphingomonas*, respectively

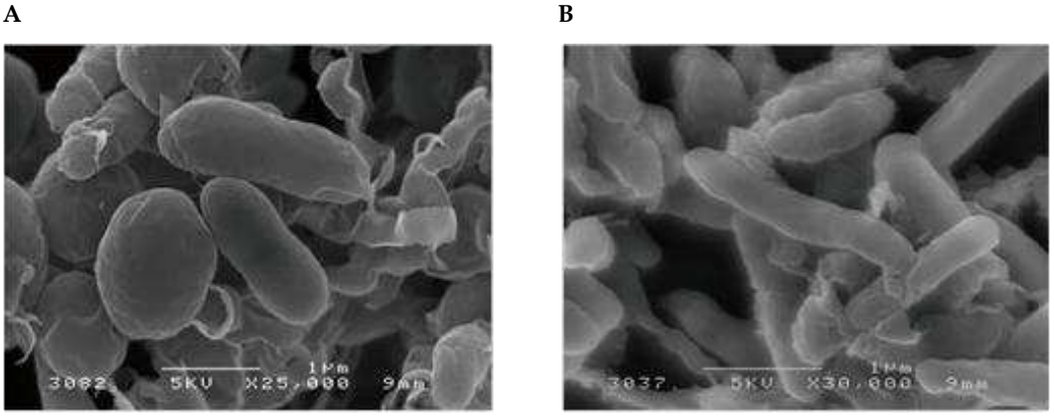

Figure 5. SEM micrographs of TCEP- and TDCPP-degrading bacteria *Sphingobium* sp. strain TCM1 (A) and *Sphingomonas* sp. stain TDK1 (B).

2.1.2.3. Degradation ability of TCEP and TDCPP-degrading bacteria

Both strains completely degraded 20 μM of TDCPP within 6 h (Fig. 6A and B). Strain TDK1, however, was 48 times less effective in degrading TCEP than TCM1 (TCEP degradation time was 144 h for TDK1, versus 3 h for TCM1) (Fig. 6A and B). During the degradations, 1,3-DCP and 2-CE were detected in the cultures of both strains and were not further degraded (Fig. 6C and D). These results showed that the strains degrade the compounds by hydrolyzing their phosphotriester bonds. To date, TCM1 and TDK1 are the only isolated microorganisms reported to degrade the persistent PFRs.

We then analyzed whether the strains can degrade other PFRs by utilizing them as sole phosphorus source. Both strains grew on tris(2,3-dibromopropyl) phosphate, tricresyl and triphenyl phosphates. Stain TDK1 did not grow on all trialkyl phosphates tested, whereas

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strain TCM1 grew moderately on tributyl phosphate and slightly on tris(2-butoxyethyl) phosphate, triethyl phosphate and trimethyl phosphate. These results demonstrate that the strains can degrade not only TDCPP and TCEP but also other PFRs, and that the strains have different substrate specificity for trialkyl phosphates.

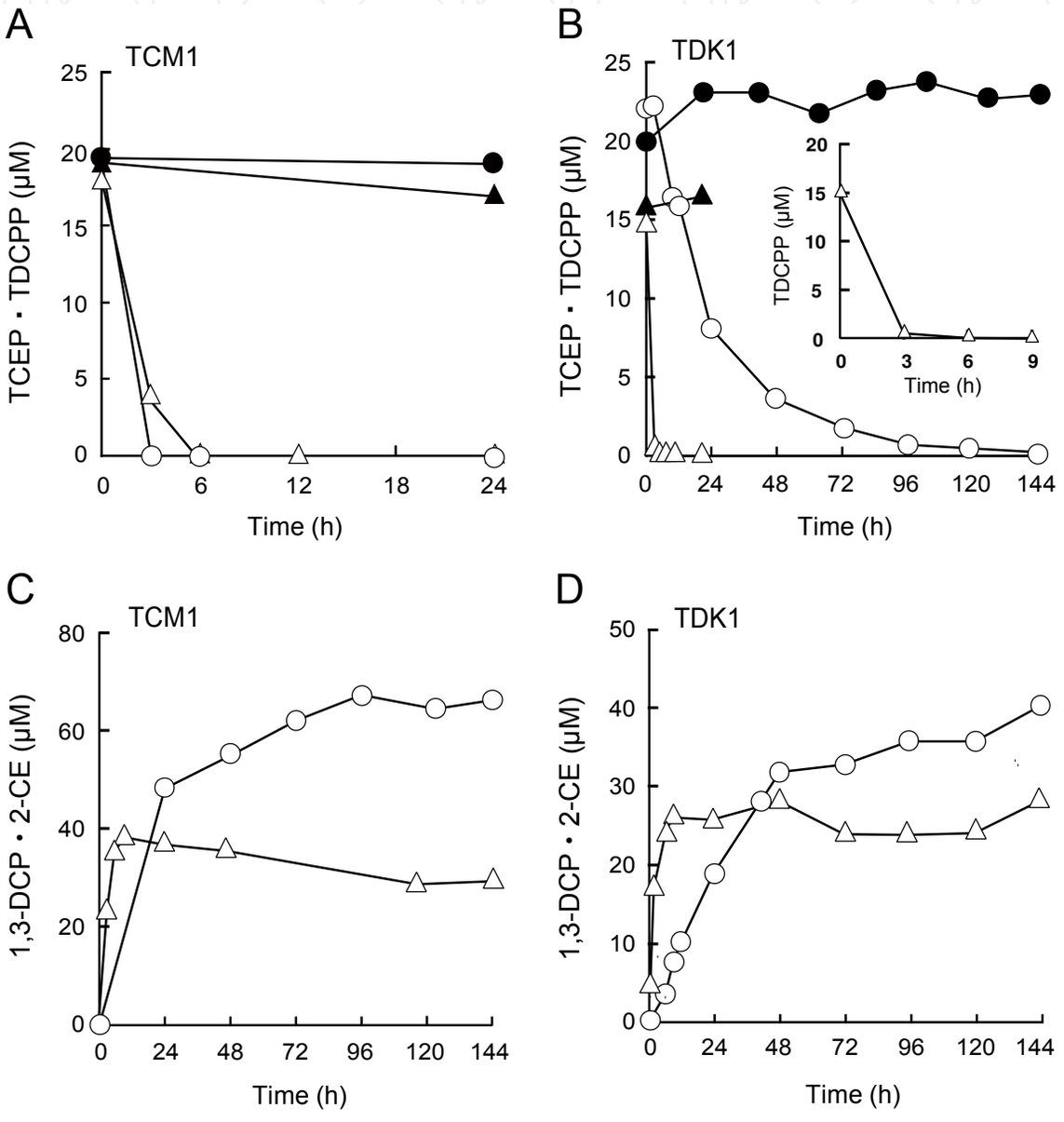

Figure 6. Degradation of TDCPP and TCEP by strains TCM1 (A) and TDK1 (B) and generation of 2-CE and 1,3-DCP (C and D). The cultivations were performed aerobically at 30°C in a minimal medium containing 20 μM of TCEP or TDCPP as the sole phosphorus source. (A and B) Open circles and triangles represent the concentrations of TCEP and TDCPP, respectively, and their filled forms represent concentrations for autoclaved control cells. (C and D) Open circles and triangles represent the concentrations of 2-CE and 1,3-DCP, respectively. Each data point represents the mean of at least two independent determinations.

2.2. Microbial detoxification of TDCPP and TCEP by two bacterial strains

We have successfully isolated TCEP- and TDCPP-degrading bacteria. However, neither strain can degrade the resulting toxic and persistent metabolites 2-CE and 1,3-DCP. Elimination of the metabolites is required before the strains can be used to degrade TDCPP and TCEP in practice. Fortunately, bacteria with chloroalcohol-degrading ability have been well-documented. We thus attempted to completely detoxify the PFRs by combining strain TCM1 with bacteria capable of degrading the chloroalcohols (Takahashi et al., 2012a; Takahashi, et al., 2012b).

2.2.1. Microbial detoxification of TDCPP using Sphingobium sp. strain TCM1 and Arthrobacter sp. strain PY1

Several 1,3-DCP-degrading bacteria have been reported, including *Arthrobacter* sp. strains PY1 (Yonetani et al., 2004) and AD2 (van den Wijngaard et al., 1991), *A. erithii* H10a (Assis et al., 1998), *Agrobacterium radiobacter* strain AD1 (van den Wijngaard et al., 1989), and *Corynebacterium* sp. strain N-1074 (Nakamura et al., 1991). Of these, *Arthrobacter* sp. strain PY1 exhibits high 1,3-DCP degradation ability. Therefore, we attempted to detoxify TDCPP by cohabitation of strain TCM1 and *Arthrobacter* sp. PY1 in a resting cell reaction (Fig. 7) (Takahashi et al., 2012a).

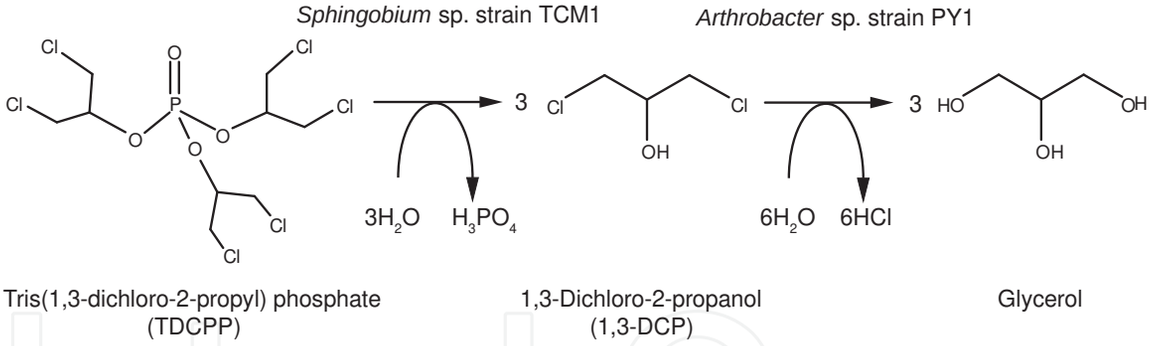

Figure 7. Complete detoxification of TDCPP by *Sphingobium* sp. strain TCM1 and 1,3-DCP-degrading bacterium *Arthrobacter* sp. strain PY1.

2.2.1.1. Freezing and lyophilization of strains TCM1 and PY1 cells

For resting cell preparation, we first examined the effect of freezing and lyophilization on the activity of strains TCM1 and PY1. The TDCPP-hydrolyzing activity of strain TCM1 intact cells was 1.07 μmol h^{-1} OD_{660}^{-1}, whereas respective activities of frozen and lyophilized cells were 0.90 and 0.84 μmol h^{-1} OD_{660}^{-1}. On the other hand, the 1,3-DCP-dehalogenating activity of strain PY1 intact cells was 0.22 μmol h^{-1} OD_{660}^{-1}, with respective frozen and lyophilized cell activities of 0.23 and 0.26 μmol h^{-1} OD_{660}^{-1}. These results reveal that freezing and lyophilization treatments cause no significant decline in degradation activities of the strains.

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2.2.1.2. Optimum TDCPP and 1,3-DCP degradation conditions of strains TCM1 and PY1

We then determined the optimum temperature and pH for lyophilized cell activity (Fig. 8). At pH 9.0 for strain TCM1 and pH 8.5 for strain PY1, the highest activity of TCM1 and PY1 cells occurred at 30°C (2.53 μmol h^{-1} OD_{660}^{-1}) and 35°C (1.31 μmol h^{-1} OD_{660}^{-1}), respectively (Fig. 8A). At 30°C, the highest activity of TCM1 and PY1 cells occurred at pH 8.5 (2.48 μmol h^{-1} OD_{660}^{-1}) and pH 9.5 with 50 mM Tris-H_2SO_4 (0.95 μmol h^{-1} OD_{660}^{-1}), respectively (Fig. 8B). We thus established the optimum temperature as 30°C and 35°C and the optimum pH as 8.5 and 9.5 for strains TCM1 and PY1, respectively.

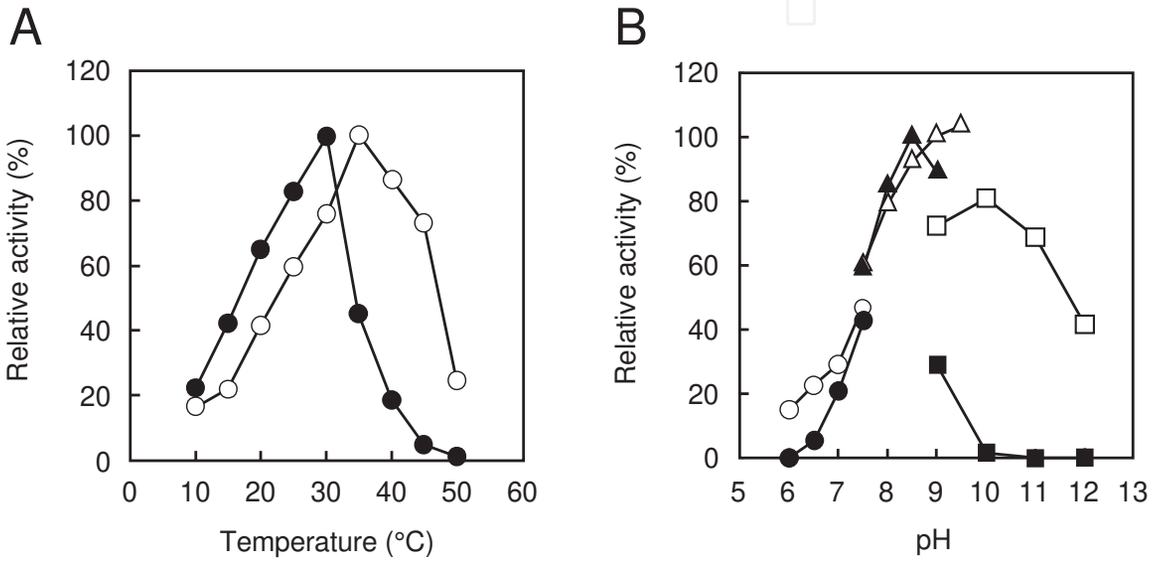

Figure 8. Effect of temperature and pH on the degradation activity of strains TCM1 and PY1. (A) effect of temperature: TDCPP hydrolyzation activity of strain TCM1 cells (closed circle) and 1,3-DCP dehalogenation activity of strain PY1 cells (open circle) were, respectively, assayed in 50 mM Tris-H_2SO_4 buffer (pH 9.0) and 50 mM Tris-H_2SO_4 buffer (pH 8.5). (B) effect of pH: TDCPP hydrolyzation activity of strain TCM1 cells (closed symbols) and 1,3-DCP dehalogenation activity of strain PY1 cells (open symbols) was assayed at 30°C in 50 mM MOPS-NaOH buffer (circle, pH 6.0-7.5), Tris-H_2SO_4 buffer (triangle, pH 7.5-9.5), and glycine-NaOH buffer (square, pH 9.0-12.0). Each datum represents means of two independent determinations.

2.2.1.3. Complete detoxification of TDCPP by mixed bacteria cells

Based on the optimum conditions, we set the reaction temperature to 30°C and pH to 9.0 (50 mM Tris-H_2SO_4) for TDCPP detoxification by mixed bacteria (Fig. 9). Under these conditions, the respective activities of strains TCM1 and PY1 were 2.21 and 0.92 μmol h^{-1} OD_{660}^{-1}. In the detoxification reaction using a mixture of TCM1 and PY1 cells (OD_{660} 0.05 and 0.2, respectively), approximately 50 μM of TDCPP disappeared within 1 h, and 1,3-DCP and chloride ions were formed to levels of approximately 100 and 120 μM, respectively, after 2 h (Fig. 9A). This result suggests incomplete detoxification of TDCPP due to low 1,3-DCP dehalogenation activity. Increasing the strain PY1 population to an OD_{660} of 4.0 decreased the TDCPP hydrolyzation rate of TCM1 cells, but completely eliminated the resulting 1,3-DCP after 10 h (Fig. 9B). At the same time, chloride ion concentration had reached its theoretical value ex-

pected from the initial TDCPP concentration, demonstrating that complete detoxification of TDCPP is achievable using strains TCM1 and PY1.

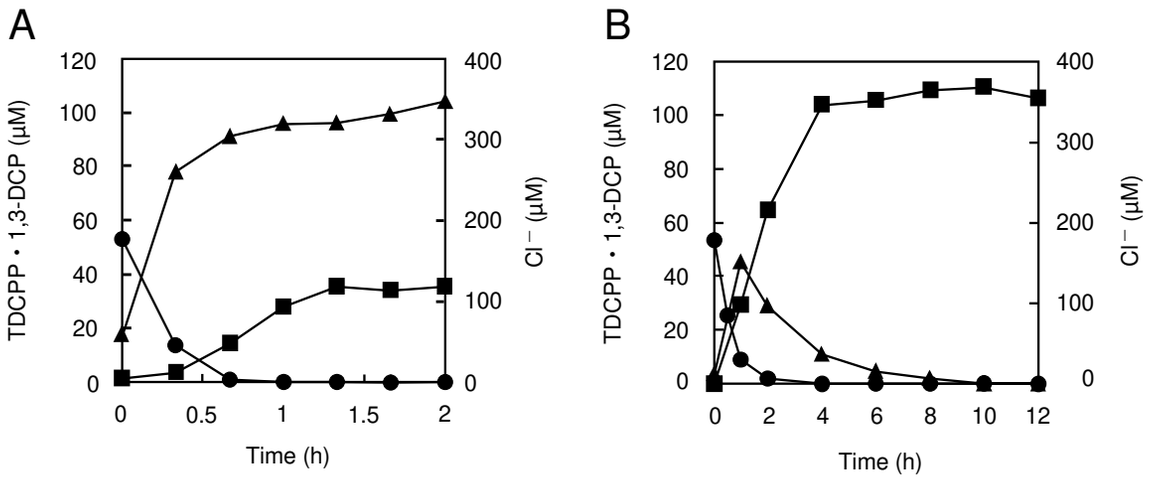

Figure 9. Complete detoxification of TDCPP by the mixed resting cells of strains TCM1 and PY1. The reactions were performed at 30°C with 50 μM TDCPP in 50 mM Tris - H_2SO_4 buffer (pH 9.0), and TDCPP (circles), 1,3-DCP (triangles) and chloride ion (squares) were determined. Cell concentrations of strains TCM1 and PY1 for each reaction were, respectively, OD_{660} of 0.05 and 0.2 (A) and 0.04 and 4.0 (B). Each datum represents means of two independent determinations.

2.2.2. Microbial detoxification of TCEP using Sphingobium sp. strain TCM1 and Xanthobacter autotrophicus strain GJ10

Several 2-CE-degrading bacteria have been reported, including *Xanthobacter autotrophicus* strain GJ10 (Janssen et al., 1985), *Pseudomonas putida* strain US2 (Strotmann et al., 1990) and *P. atutzeri* strain JJ (Dijk et al., 2003). Among these, the degradation of 2-CE by *X. autotrophicus* strain GJ10 has been well characterized. Therefore, we attempted to detoxify TCEP by co-habitation of strain TCM1 and *X. autotrophicus* strain GJ10 (Fig. 10) (Takahashi et al., 2012b).

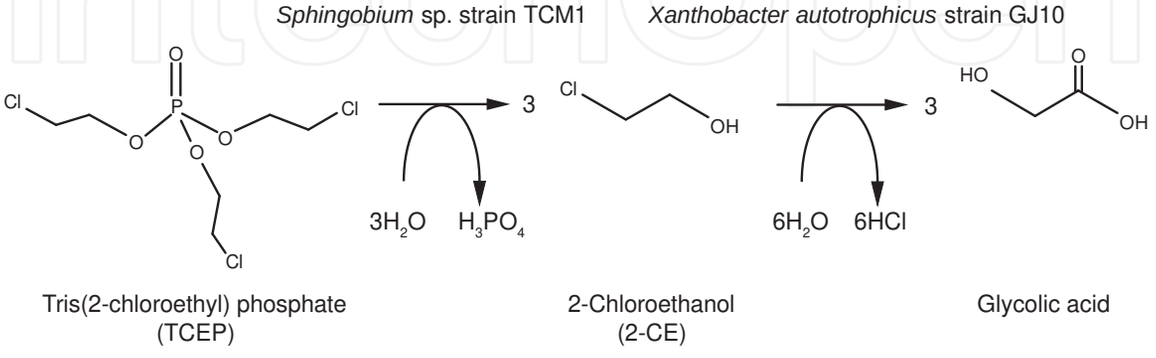

Figure 10. Complete detoxification of TCEP by *Sphingobium* sp. strain TCM1 and 2-CE-degrading bacterium *Xanthobacter autotrophicus* strain GJ10.

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2.2.2.1. Optimum TCEP degradation condition of strain TCM1

We first determined the optimum temperature and pH for TCEP degradation by strain TCM1 in a resting reaction using lyophilized cells. At pH 7.4, the highest activity was obtained at 30°C (14.1 nmol min^{-1} OD_{660}^{-1}). Maintaining this temperature and varying the pH, the highest activity was recorded at pH 8.5 (14.6 nmol min^{-1} OD_{660}^{-1}). These optimum conditions were identical to those for TDCPP, suggesting that the same enzyme(s) might be involved in the degradation of both compounds.

Under the optimum conditions, TCM1 cells completely eliminated 10, 20 and 50 μM of TCEP within 3 h, but the generated 2-CE was approximately 50% of its theoretical value based on the initial TCEP concentrations (Fig. 11). Phosphotriesterase that can hydrolyze organophosphorus pesticides structurally similar to TCEP, such as chlorpyrifos, require two zinc ions for catalysis, and enzyme activity can be maximized by replacing Zn^{2+} with Co^{2+} (Omburo et al., 1992). A bacterial phosphodiesterase that can hydrolyze alkyl phosphodiesters similarly requires divalent metals (Gerlt & Wan, 1979). We therefore examined the effect of Co^{2+} as well as cell amount on TCEP hydrolysis (Fig. 11). In the reaction using approximately 10 μM of TCEP without Co^{2+}, 2-CE reached 21.2 μM (OD_{660} of 0.8) after 3 h. Addition of 50 μM Co^{2+} resulted in an increase of 2-CE to 32.3 μM, equivalent to the theoretical value of 30 μM (Fig. 11B). These results showed that complete hydrolysis can be achieved at an OD_{660} of 0.8 with 50 μM of Co^{2+}.

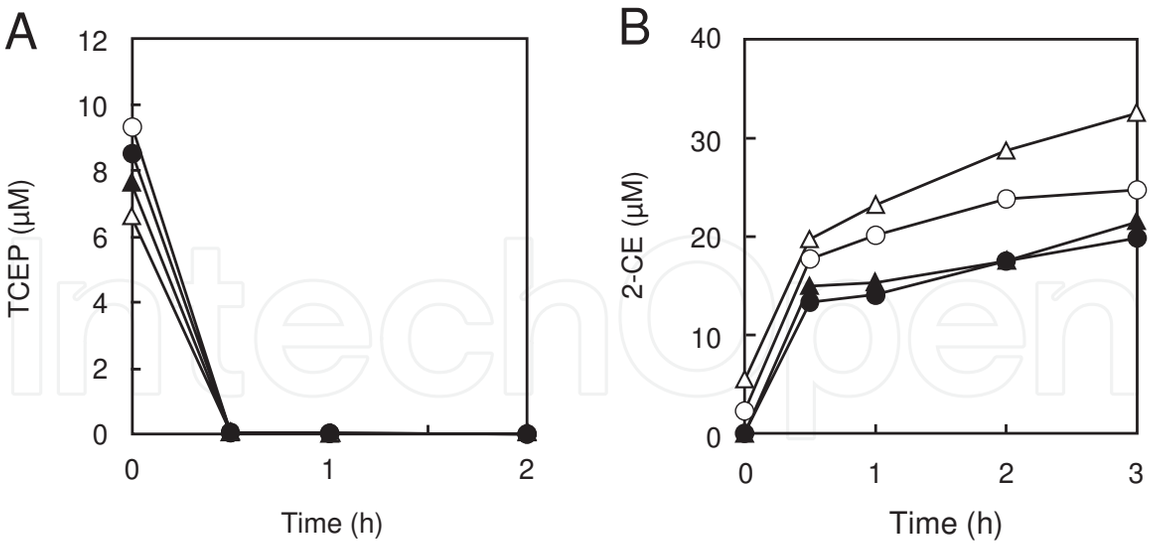

Figure 11. Effect of Co^{2+} and cell amount on TCEP hydrolysis by strain TCM1-resting cells. The reactions were performed at 30°C using the resting cells at OD_{660} of 0.4 (circles) or 0.8 (triangles) with (open symbols) or without (closed symbols) 50 μM Co^{2+} in 50 mM Tris-H_2SO_4 buffer (pH 8.5) containing 10 μM TCEP, and TCEP (A) and 2-CE (B) were determined. Each datum represents the mean of two independent determinations. The inconsistency of the initial concentrations of TCEP at zero time with the set-up ones was mainly attributed to reaction progress in several minutes to stop the reaction.

2.2.2.2. Optimum 2-CE degradation condition of strain GJ10

We prepared resting cells of intact, frozen and lyophilized cells of *X. autotrophicus* strain GJ10 and examined their 2-CE degradation activity. Activity was detected only in frozen cells at 4.93 pmol min^{-1} OD_{450}^{-1}, four orders lower than the TCEP degradation activity of strain TCM1. This low 2-CE degradation activity might be attributable to the lack of coenzyme regeneration of enzymes involved in the degradation process. We next examined 2-CE degradation in a growing cell reaction. The growing cells completely degraded approximately 180 μM of 2-CE within 24 h. The degradation ability was estimated to be a minimum of 7.5 μM h^{-1}, comparable to the TCEP degradation ability of strain TCM1-resting cells (approximately 10 μM h^{-1}). This result shows that growing cells of strain GJ10 can degrade 2-CE effectively.

2.2.2.3. Complete detoxification of TCEP by two bacterial strains

Based on the results described above, we examined whether combining TCEP hydrolysis by TCM1 resting cells and 2-CE degradation by GJ10 growing cells would completely detoxify TCEP (Fig. 12). TCM1 resting cells abolished 9.6 μM of TCEP within 4 h, releasing 2-CE at 29.0 μM, equivalent to that estimated from the initial TCEP concentration, and consistent with complete TCEP hydrolysis (Fig. 12A and B). The generated 2-CE was abolished by GJ10 growing cells within 48 h, and chloride ion concentration reached 30.2 μM after 144 h, equivalent to that estimated from the generated 2-CE (Fig. 12C and D). Taken together, these results demonstrate that complete detoxification of TDCPP can be achieved using strains TCM1 and GJ10.

3. Concluding remarks

We have successfully isolated two novel bacterial strains capable of degrading the persistent and potential toxic PFRs, TCEP and TDCPP, which have become worldwide environmental contaminants. The two strains TCM1 and TDK1 belong to *Sphingobium* sp. and *Sphingomonas* sp. respectively. The strains are the first microorganisms reported to degrade the persistent PFRs. They degrade the compounds by hydrolyzing their phosphotriester bonds to produce metabolites 1,3-DCP from TDCPP and 2-CE from TCEP, which are themselves toxic and non-self-biodegradable. In a successful attempt to completely detoxify the FPRs, we combined TCM1 with the 1.3-DCP-degrading bacterium *Arthrobacter* sp. strain PY1 (for TDCPP degradation), and with the 2-CE-degrading bacterium *X. autotrophicus* strain GJ10 (for TCEP degradation). This is the first description of microbial FPR detoxification. The bacteria and the microbial detoxification techniques may prove useful for the bioremediation of sites contaminated with intractable compounds. Further studies on the PFRs-degrading bacteria as well as the chloroalcohols-degrading bacteria, and on the detoxification techniques, could help to establish more efficient detoxifications, and could also provide novel insights into microbial degradation of organophosphorus compounds. We are now working towards elucidating the enzymes and the genes involved in the degradation processes.

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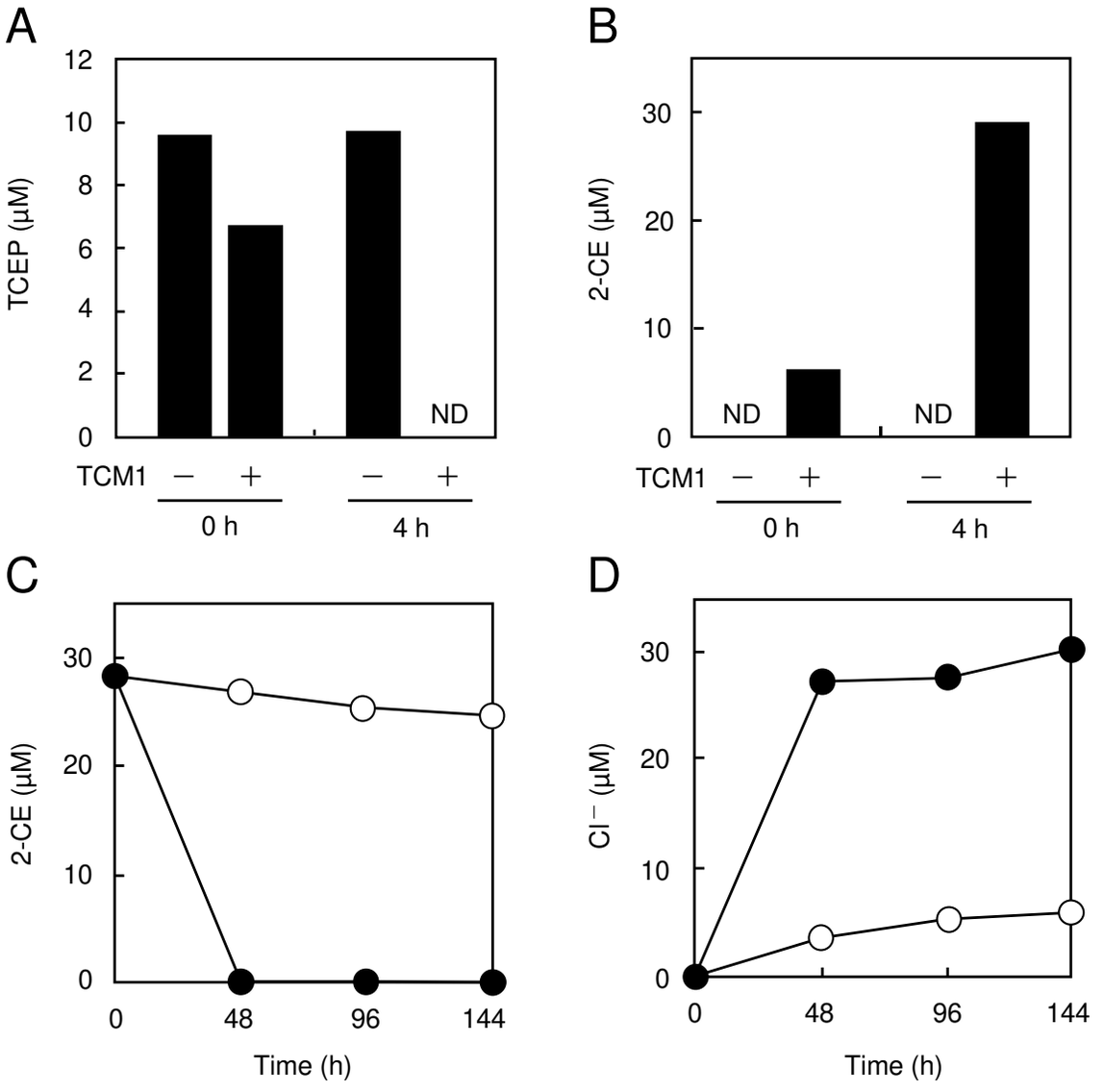

Figure 12. Complete detoxification of TCEP by *Sphingobium* sp. strain TCM1-resting cell reaction (A and B) and the following *X. autotrophicus* GJ10-growing cell reaction (C and D). The resting cell reaction was performed at 30°C with (+) or without (-) strain TCM1 cells at OD_{660} of 0.8 in 50 mM Tris-H_2SO_4 buffer (pH 8.5) containing 10 μM TCEP and 50 μM Co^{2+}, and TCEP (A) and 2-CE (B) were determined. The growing cell reaction was performed at 30°C with (closed symbols) or without (open symbols) strain GJ10 cells in a medium containing the generated 2-CE as the sole carbon source, and 2-CE (C) and chloride ion (D) was determined. ND means not detected. Each datum represents the mean of two independent determinations.

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