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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).

TDCPP is a viscous colorless to light yellow liquid and is produced by the epoxide opening of epichlorohydrin in the presence of phosphorus oxychloride (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 manufacturers 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 TDCPP 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
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                                  |
| **Synonym:**                                      | **115-96-8**                                 |
| Tris(1,3-dichloro-2-propyl) phosphate              | Tris(2-chloropropyl) phosphate               |
| Tris-(2-chloro-,1-chloromethyl-ethyl)-phosphate    | Tris(2-chloroethyl) phosphate                |
| 1,3-dichloro-2-propanol phosphate                 | Tris(β-chloroethyl) phosphate               |
| Phosphoric acid, tris(1,3-dichloro-2-propylester)  | Phosphoric acid, tris(2-chloroethyl) ester   |
| Tris(1,3-dichloroisopropyl) phosphate              | Tris(2-chloroethyl) orthophosphate           |
| Tris(1-chloromethyl-2-chloroethyl) phosphate       | Tris(chloroethyl) phosphate                  |
| Tri(β, β'-dichloroisopropyl) phosphate             |                                             |

**Table 1.** General aspect of Tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and tris(2-chloropropyl) 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 to 150 ng m$^{-3}$ in Sweden houses, and in Belgium office and stores, they have reached 73 ng m$^{-3}$ (Table 2). In outdoor air, TDCPP levels near a main road in Sweden ranged from <0.04-0.072 m$^{-3}$, 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$^{-3}$ | office and store | Norway | SFT, 2008 |
|             | <0.2-150 ng m$^{-3}$ | home, cinema, university, hospital, hotel, prison, library, office shops | Sweden | Marklund et al., 2005a |
|             | <0.3-7 ng m$^{-3}$ | lecture and computer hall, electronic dismantling facility recycling plant | Sweden | Staaf & Ostman, 2005 |
|             | <73 ng m$^{-3}$ | work place | Belgium | Bergh et al., 2011 |
|             | <61.4 ng m$^{-3}$ | house | Japan | Kanazawa et al., 2010 |
| Environment   | Concentration | Location                        | Country       | Reference                          |
|---------------|---------------|---------------------------------|---------------|------------------------------------|
| Indoor dust   | 0.2-67 μg g⁻¹ | home, cinema, university,       | Sweden        | Marklund et al., 2003              |
|               |               | hospital, hotel, prison, library, office shops |               |                                    |
| <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 | nearby main road               | Sweden         | Marklund et al., 2003              |
|                | <0.04-0.14 ng m⁻³ | remote area from main road     | Sweden         | Marklund et al., 2003              |
| n.d.-5 pg m⁻³   | sea           | Arctic ocean                    | Moller et al., 2012    |
| 16-52 pg m⁻³    | sea           | Japan                           | Moller et al., 2012    |
| 5-8 pg m⁻³      | sea           | Northern pacific ocean         | Moller et al., 2012    |
| 49-780 pg m⁻¹³  | sea           | East Indian archipelago,        | Moller et al., 2012    |
|                |               | Philippine sea                  |               |                                    |
| n.d.-220 pg m⁻¹³| sea           | Indian ocean                    | Moller et al., 2012    |
| 80 pg m⁻¹³      | 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-Carbillo et al., 2007     |
|                | <3.0-19 ng L⁻¹| river                           | Austria        | Martinez-Carbillo 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      | Germany        | Andresen & Bester, 2006            |
|                | <250 ng L⁻¹   | water after drinking water      | United States  | Stackelberg et al., 2004           |
|               |               | treatment                       |               |                                    |
|               |               |                                  |               |                                    |
| 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 | Switzerland | 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 |
| 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 | cod liver and mussel | Norway | SFT, 2008 |
|             | 49-140 ng g⁻¹ | freshwater fishes close to sources | Norway | Sundkvist et al., 2010 |
|             | 16.1-5.3 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 inside an office furnished with linoleum floor and a new photocopier (Table 3). In outdoor air, it can reach 6.2 ng m⁻³ beside a main road, but remote areas harbor less than 0.2 ng m⁻³, implicating...
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\(^{-3}\) | office and store | Norway | SFT, 2008 |
| 3, 9 ng m\(^{-3}\) | lecture room and kindergarten | Sweden | Tollback et al., 2006 |
| 0.4-730 ng m\(^{-3}\) | home, cinema, university, hospital, hotel, prison, library, office shops | Sweden | Marklund et al., 2005a |
| <0.3-10 ng m\(^{-3}\) | Lecture and computer hall, electronic dismantling facility recycling plant | Sweden | Staaf & Ostman, 2005 |
| <22 ng m\(^{-3}\) | car, theater, furniture store, office and electronics store | Sweden | Hartmann et al., 2004 |
| 3-15 ng m\(^{-3}\) | lecture room and office room | Sweden | Bjorklund et al., 2004 |
| <297 ng m\(^{-3}\) | house | Japan | Kanazawa et al., 2010 |
| <136 ng m\(^{-3}\), <421 ng m\(^{-3}\) | house and office | Japan | Saito et al., 2007 |
| 1.2 ng m\(^{-3}\) | newly constructed house | Japan | Saito et al., 2007 |
| <28 ng m\(^{-3}\) | home | Belgium | Bergh et al., 2011 |
| 7.8-230 ng m\(^{-3}\) | day care center | Belgium | Bergh et al., 2011 |
| Environment     | Concentration | Location                                      | Country   | Reference                      |
|-----------------|---------------|-----------------------------------------------|-----------|--------------------------------|
| Indoor dust:    | <140 ng m⁻³  | work place                                    | Belgium   | Bergh et al., 2011             |
|                 | 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 | home, cinema, university, hospital, hotel, prison, library, office shops | Sweden    | Marklund et al., 2003          |
|                 | <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  | nearby main road                               | Sweden    | Marklund et al., 2003          |
|                 | <0.2 ng m⁻³  | remote area from main road                     | Sweden    | Marklund et al., 2003          |
|                 | 126-585 pg m⁻³ | ocean                                          | Arctic ocean | Moller et al., 2012         |
|                 | 273-1,961 pg m⁻³ | sea                                            | Japan     | Moller et al., 2012             |
|                 | 159-282 pg m⁻³ | sea                                            | Northern pacific ocean | Moller et al., 2012         |
|                 | 19-156 pg m⁻³ | sea                                            | East Indian archipelago, Philippine sea | Moller et al., 2012         |
|                 | 46-570 pg m⁻³ | sea                                            | Indian ocean | Moller et al., 2012         |
|                 | 74 pg m⁻³     | 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-Carbello 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          |
|                 | 4,230-8,400 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    | raw water of waste disposal site               | Germany   | European Commission DG ENV, 2011 |
|                 | <312 ng L⁻¹  | raw water of waste disposal site               | Germany   | Fries & Püttmann, 2003          |
| Environment | Concentration | Location | Country | Reference |
|-------------|---------------|----------|---------|-----------|
| Drinking water: 0.74-1.7 ng L⁻¹ | water after drinking water treatment | Germany | Fries & Püttmann, 2001 |
| 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 | fish muscle and liver | Norway | SFT, 2009b |
| 13-26 ng g⁻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 |
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) | EURAR, 2009                |
|                     | NOAEL=175 mg kg$^{-1}$ day$^{-1}$ mouse (16 weeks by gavage) | EURAR, 2009                |
| Reproductive toxicity | rat and mouse                        | EURAR, 2009                |
|                     | NOAEL=175 mg kg$^{-1}$ day$^{-1}$ mouse for fertility | EURAR, 2009                |
| 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).
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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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](image-url). 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-
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 (NaH₂PO₄): 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 Sphingomonas 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          |
|---------|------|----------------------------------|
| 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.
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).

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.
2.1.2.2. 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. strain TDK1 (B).](image)

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...
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 co-habitation of strain TCM1 and Arthrobacter sp. PY1 in a resting cell reaction (Fig. 7) (Takahashi et al., 2012a).

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.
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$^{-1}$) and 35°C (1.31 μmol h$^{-1}$ OD$^{-1}$), 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$^{-1}$) and pH 9.5 with 50 mM Tris-H$_2$SO$_4$ (0.95 μmol h$^{-1}$ OD$^{-1}$), 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$_2$SO$_4$ buffer (pH 9.0) and 50 mM Tris-H$_2$SO$_4$ 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$_2$SO$_4$ 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$_2$SO$_4$) 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$^{-1}$. 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$_2$SO$_4$ 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.
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 OD₆₆₀⁻¹). Maintaining this temperature and varying the pH, the highest activity was recorded at pH 8.5 (14.6 nmol min⁻¹ OD₆₆₀⁻¹). 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 Zn²⁺ with Co²⁺ (Omburo et al., 1992). A bacterial phosphodiesterase that can hydrolyze alkyl phosphodiester similarly requires divalent metals (Gerlt & Wan, 1979). We therefore examined the effect of Co²⁺ as well as cell amount on TCEP hydrolysis (Fig. 11). In the reaction using approximately 10 μM of TCEP without Co²⁺, 2-CE reached 21.2 μM (OD₆₆₀ of 0.8) after 3 h. Addition of 50 μM Co²⁺ 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 OD₆₆₀ of 0.8 with 50 μM of Co²⁺.

Figure 11. Effect of Co²⁺ and cell amount on TCEP hydrolysis by strain TCM1-resting cells. The reactions were performed at 30°C using the resting cells at OD₆₆₀ of 0.4 (circles) or 0.8 (triangles) with (open symbols) or without (closed symbols) 50 μM Co²⁺ in 50 mM Tris-H₂SO₄ 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 \textit{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 \textit{Sphingobium} sp. and \textit{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 PFRs, we combined TCM1 with the 1,3-DCP-degrading bacterium \textit{Arthrobacter} sp. strain PY1 (for TDCPP degradation), and with the 2-CE-degrading bacterium \textit{X. autotrophicus} strain GJ10 (for TCEP degradation). This is the first description of microbial PFR 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.
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\textsubscript{660} of 0.8 in 50 mM Tris-H\textsubscript{2}SO\textsubscript{4} buffer (pH 8.5) containing 10 μM TCEP and 50 μM Co\textsuperscript{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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Author details

Shouji Takahashi*, Katsumasa Abe and Yoshio Kera
Department of Environmental Systems Engineering, Nagaoka University of Technology, Kami‐mitomioka, Nagaoka, Niigata, Japan

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