

MICROBIAL ENRICHMENT METHODOLOGY FOR BIODEGRADATION OF POLYBROMINATED DIPHENYL ETHERS, RECALCITRANT XENOBIOTICS IN SEWAGE SLUDGE IN ITALY

Eudes LANCIOTTI¹, Arianna PORFIDO¹, Tania MARTELLINI², Alessandra CINCINELLI², Giuseppe MANCINI³

- (1) Department of Public Health, Hygiene section, University of Firenze, Italia, email: eudes.lanciotti@unifi.it, (corresponding author).
(2) Department of Chemistry, University of Firenze, Italia, email: alessandra.cincinelli@unifi.it
(3) Department of civil and Environmental Engineering, University of Catania, Italia, email: gmancini@dica.unict.it

ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are flame retardants present in several commercial products and furniture including building materials, electronics, furnishings, motor vehicles, airplanes, plastics, polyurethane foams, and textiles. They have been used for three decades and very little is known about their fate in the environment and their susceptibility to aerobic biotransformation. Aims of this study were to determine the PBDE concentrations in sludge samples collected in different waste water treatment plants (WWTPs) in Italy and to investigate the ability of aerobic microbial strains to biodegrade PBDEs. All samples were analysed by a gas chromatograph Agilent 6890 connected to a mass spectrometer Agilent 5973 Network (Agilent Technologies) with a negative chemical ionization ion source (GCMS-NCI). Microbial degradation sludge was made by two bacterial aerobic strains (*Rhodococcus jostii* (RHA1) e *Burkholderia xenovorans* (DSMZ 17367) added to microbial flora sludge on culture medium and on bench scale pilot plant. Primary sludge samples were collected from 8 WWTPs located in the North and Centre area of Italy between June 2007 and March 2010 treating different effluents (municipal, industrial and/or combined). The capacity of the WWTP investigated ranged from 35000 to 1250000 population equivalents and from 12500 m³/day to 432000 m³/day. PBDE concentration data for sludge samples collected from the 8 wastewater treatment plants showed total tri- to hepta- BDE concentrations (sum of significant congeners BDE 28, 47, 99, 153, 54 and 183) ranging from 19.48 to 99.89 (median 45.73 ± 26.61) ng/g d.w. and deca-BDE (BDE209) concentrations from 131 to 7122 (median 2741 ± 2849) ng/g d.w.. BDE 209 dominated the congener profile, contributing between 73% and 99% of the total BDE (mean: 90.0 ± 9.9 %), suggesting the present use of this technical formulation in the investigated WWTP. Results experiments on culture medium are not yet available. Experiments on sludge in bench scale pilot plant produced considerable results demonstrating microbial attack of commercial deca-BDE and environmental BDE with six bromines or less: halving penta-BDE mixture (929,4 Vs 463,5 ng/g), great reduction of octa-BDE mixture (258,5 Vs 105,5 ng/g) and increase mono-BDE mixture (slr Vs 48,8 ng/g) .

1 INTRODUCTION

Microbial biodegradation of persistent xenobiotics represents a necessity of industrialized countries. Among xenobiotics emerges polybrominated diphenyl ethers (PBDEs), commonly used as flame retardants in a wide array of products, including building materials, electronics, furnishings, motor vehicles, airplanes, plastics, polyurethane foams, and textiles. PBDEs are class of flame retardants that have been used for more decades, global production of PBDEs is approximately 40,000 tons per year. Commercial PBDE formulations are named by their average degree of bromination: penta- (mostly penta- and tetra-BDE congeners), octa- (mostly octa- and hepta-BDE congeners) and deca-BDE mixture. These congeners persist in the environment and biota, and thereby transferred to humans via the food chain. In environment and biota, PBDE more highly brominated congeners (such as deca-, hepta-, esa-BDE) are biotransformed in minor brominated congeners (such as penta-, tetra-, mono-BDE), that result more toxic (Denef et al, 2002; Gerecke et al, 2005; La Guardia et al. 2007; Law et al., 2006; Schecter et al., 2004).

Increasing concentrations in environmental samples and human tissues have focused worldwide attention on the potential health effects of PBDEs. Data from animal exposures indicate an adverse effect of PBDEs: some PBDEs are toxic and others cause endocrine disruption in rats and mice (FAO/WHO, 2005; Martin et al. 2000). As of 2006, the penta- and octa-BDE mixtures have been banned and removed from production in Europe and United States (Dye et al., 2007; Birnbaum et al., 2006; Martin et al. 2000; Schecter et al., 2005; Zota et al., 2008).

Very little is known about PBDEs fate in the environment and their susceptibility to aerobic biotransformation and microbial reductive debromination. Sewage sludge produced in waste water treatment plants contains large amounts of PBDEs (Nyholm et al., 1996). In nature and in waste water treatment, PBDEs congeners are susceptible to transformation by certain species of aerobic bacteria, such as microbial flora sludge (He et al. 2006) and two known PCB-degrading bacteria: *Rhodococcus jostii* RHA1 and *Burkholderia xenovorans* LB400 (Denef et al. 2004, Goris et al. 2004, Gulari et al. 2004; Robrock et al. 2009; He et al. 2006). Aims of this study were to determine the PBDE concentrations in sludges samples collected in different waste water treatment plants (WWTPs) in Italy and to investigate the ability aerobic microbial strains capable to biodegrade PBDEs. Thus two known PCB-degrading bacteria, *Rhodococcus jostii* RHA1 and *Burkholderia xenovorans*, were added to microbial flora sludge on culture medium and on bench scale pilot plant, and exposed to PBDE congeners ranging from tetra- to deca-BDEs.

2 METHODS AND MATERIALS

Primary sludge samples were collected from 8 WWTPs located in the North (5) and Centre (3) area of Italy between June 2007 and March 2010 treating different effluents (municipal, industrial and/or combined). The capacity of the WWTP investigated ranged from 35000 to 1250000 population equivalents and from 12500 m³/day to 432000 m³/day (see WWTP characteristics in the table 1).

Table I. Capacity of WWTPs

WWTP	m ³ /day	Population Equivalent
A	95500	420000
B	30000	120000
C	12500	35000
D	432000	1250000
E	345000	1050000
F	35000	160000
G	130000	750000
H	80000	600000

PBDEs extraction. Freeze dried sludge samples were weighted (about 5g), homogenized and sieved (2 mm); the samples were spiked with recovery standards, then, were Soxhlet extracted using 200 ml dichloromethane for 16 h. The extracts were reduced in volume using rotoevaporation and cleaned up using multi-layer silica column. The multi-layer silica column had three pretreated silica layers: acid silica, basic silica, AgNO₃ silica layer and 10 g of anhydrous sodium sulfate on the top; overnight (at 250°C) baked silica was shaken for 8 hours with sulfuric acid (95% to 97%), sodium hydroxide solution (1 M) or AgNO₃ solution (1.5 M). For elution were used 50 ml of cyclohexane followed by 50 ml of cyclohexane: dichloromethane (4:1 v/v). The eluents were mixed and then concentrated to 2 ml. All samples were analyzed by a gas chromatograph Agilent 6890 connected to a mass spectrometer Agilent 5973 Network (Agilent Technologies) with a negative chemical ionization ion source (GCMS-NCI). Congeners from tri- to octa-BDEs were determined using a DB-35 (30 m 0.25 mm i.d., 0.25 µm film thickness) capillary column. The used carrier gas was Helium at the constant flow rate 1.2 ml/min; for the injection was used the splitless mode with the injection volume of 1 µl at 250°C. The temperature was initially from 50°C, held for 1 min, to 130°C with a 20°C/min rate, then 5°C/min to 300°C, finally 20°C/min to 250°C; run time of 57.45 min. For the qualification of Deca-BDE was used a 15 m capillary column DB-5MS (0.25 mm i.d., 0.10 µm film thickness) and a oven temperature program that start at 90°C for 1 min, a 30°C/min up to 220°C, then 10°C/min up to 300°C and was held for 8 min; run time of 21.33 min.

BDE-degrading bacteria. Microbial degradation activated sludge was made by two bacterial aerobic strains (*Rhodococcus jostii* (RHA1) e *Burkholderia xenovorans* (DSMZ 17367) added to microbial flora sludge on culture medium and on bench scale pilot plant (Bedard et al, 1986; Deneff et al. 2004; Goris et al, 2004; Gulari et al., 2004; Seto et al., 1995)

On culture medium, the strains were grown in a mineral medium modified from that described in Sharp et al. 2005 and contains: 10g/L NaNO₃, 1.7 g/L K₂SO₄, 0.37 g/L MgSO₄·7H₂O, 0.086 g/L CaSO₄·2H₂O, 5.3 g/L KH₂PO₄, 10.6 g/L K₂HPO₄, 5.74 mg/L ZnSO₄·7H₂O, 3.38 mg/L MnSO₄·H₂O, 1.24 mg/L H₃BO₃, and 5 mg/L CuSO₄·5H₂O.

On bench scale pilot plant, three types of experiments were performed: (1) using an activated sludge enriched with the two bacterial strains from collections (*Burkholderia xenovorans* and *Rhodococcus jostii*) at a concentrations of 10⁴ CFU/ml, oxygenated for 96 h and supplied with nutrients; (2) in batch experiment with activated sludge enriched with two bacterial strains from the collection (*B. xenovorans* and *R. jostii*), at concentrations of 10⁵ CFU/ml, oxygenated for 48 h, (3) in batch experiment with an activated sludge as it is (with only the indigenous microflora and without bacterial enrichment), oxygenated for 48 h.

3 RESULTS AND DISCUSSION

PBDEs in primary sludges. PBDE concentration data for primary sludge samples collected from the 8 wastewater treatment plants and analysed by GC-NCI-MS showed total tri- to hepta- BDE concentrations (sum of significant congeners BDE 28, 47, 99, 153, 54 and 183) ranging from 19.48 to 99.89 (median 45.73 ± 26.61) ng/g d.w. and deca-BDE (BDE209) concentrations from 131 to 7122 (median 2741 ± 2849) ng/g d.w. BDE 209 dominated the congener profile, contributing between 73% and 99% of the total BDE (mean: 90.0 ± 9.9 %), suggesting the present use of this technical formulation in the investigated WWTP.

Figure 1 reports concentrations of penta, octa and deca-BDE in sludge samples. The results were comparable to the reported in literature for European WWTPs treating similar inlets. In particular, the mixed sewage plant of northern Italy (5 plants in Milano-Varese area) had the following deca and penta-BDE values: water input min 1.5-max 125 ng/L (deca BDE, 209); 0.5 min-max 14 ng/L (for penta BDE, 99); water output: min 1-max 37 ng/L (deca) min 0.2-max 0.6 ng/L (penta). While mixed sewage plant of the center area (3 plants in Firenze-Prato-Pistoia area) had the following deca and penta-BDE values: water input max 2100 ng/L (deca), max 12 ng/L (penta), water output max 300 ng/L (deca), max 2 ng/L (penta). This results were comparable to that reported in literature ((He et al. 2006; La Guardia et al., 2007; Nyholm et al. 1996).

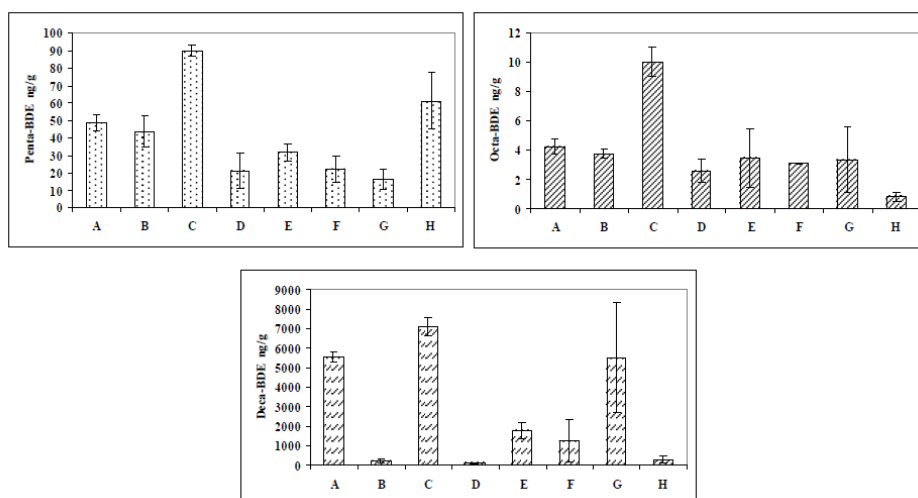


Figure 1. PBDE mixture concentrations in the sludge samples.

Microbial PBDE degradation. Results experiments on culture medium are not yet available. Experiments on bench scale pilot plant produced considerable results demonstrating microbial attach of commercial deca-BDE on sludge: halving penta-BDE mixture (929,4 vs 463,5 ng/g), great reduction of octa-BDE mixture (258,5 vs 105,5 ng/g) and increase mono-BDE mixture (bdl vs 48,8 ng/g).

In the experiments on bench scale pilot plant, for the analytical determination of BDE these two steps can be distinguished: (a) activated sludge as it is and before microbial enrichment (time-0), (b) activated sludge after microbial enrichment (time-1), with oxygen in the oxidation tank for 96 h. In the experiments on bench scale pilot plant had been

determined the values of the major BDE congeners that occur in wastewater, following the degradation of deca-BDE (209), and particularly in the activated sludge enriched with the two bacterial strains from collections (*Burkholderia xenovorans* and *Rhodococcus jostii*), oxygenated for 96 h (time-1). Among the main biodegradation products of deca-BDE there are mixtures octa-BDE and penta-BDE, up to mono-BDE (see Table 2). This results were comparable to that reported in literature (Denef et al. 2004; Goris et al, 2004; Gulari et al., 2004; Seto et al., 1995).

The analytical investigation on PBDE has provided the following data: at time-0 mono-BDE presents values below detection limit (bdl), while at time-1 the value is 48.8 ng/g; penta-BDE mixture, at time 0 has a value of 929.4 ng/g, while at time-1 has a value of 463.5 ng/g; the mixture octa, at time-0 has a value of 258.5 ng/g, while at time-1 has a value of 105.5 ng/g (see Figures 2 and 3).

Among the individual BDE congeners (Table 2) some important values emerge in the biodegradation degree; tetra-BDE-47 at time-0 presents value of 517.5 ng/g, that markedly decrease at time-1 with values of 107.2 ng/g; esa-BDE-154 at time-0 presents 130.4 ng/g, while at time-1 presents 68.5-ng/g; esa-BDE 156 at time-0 presents 118.9 ng/g and at time-1 77.2 ng/g; the hepta-BDE 183 at time-0 presents 258.5 ng/g, at time-1 105.5 ng/g; hepta-BDE-191 presents at time-0 258, 5 ng/g and at time-1 58.3 ng/g.

Table II. BDE congener and penta, octa and deca-BDE mixture concentrations (ng/g) in activated sludge of the bench scale pilot plant before (time 0) e after (time 1) microbial enrichment (b.d.l. = below the detection limit)

PBDEs	before microbial enrichment (time-0) (ng/g)	after microbial enrichment (time-1) (ng/g)
BDE-7	bdl	48,80
BDE15	bdl	59,77
BDE-17	87,5	68,6
BDE28	75,5	61,2
BDE-49	79,6	114,8
BDE-71	59,3	49,0
BDE47	517,5	107,2
BDE-66	76,1	67,8
BDE-100	118,2	130,0
BDE-119	20,4	18,6
BDE-99	84,2	81,0
BDE-85+126	185,5	68,2
BDE-154	130,4	68,5
BDE-153	79,1	76,8
BDE-138	73,5	67,6
BDE-156	118,9	77,2
BDE-184	85,6	74,0
BDE-183	258,5	105,5
BDE-191	258,5	158,3
mono	bdl	48,8
penta	929,4	463,5
octa	258,5	105,5

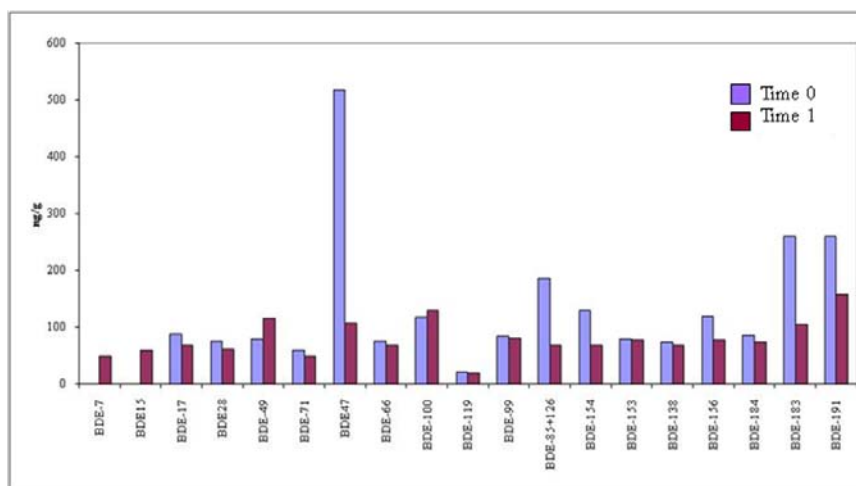


Figure 2. BDE congener concentrations (ng/g) in activated sludge of the bench scale pilot plant before (time 0) e after (time 1) microbial enrichment.

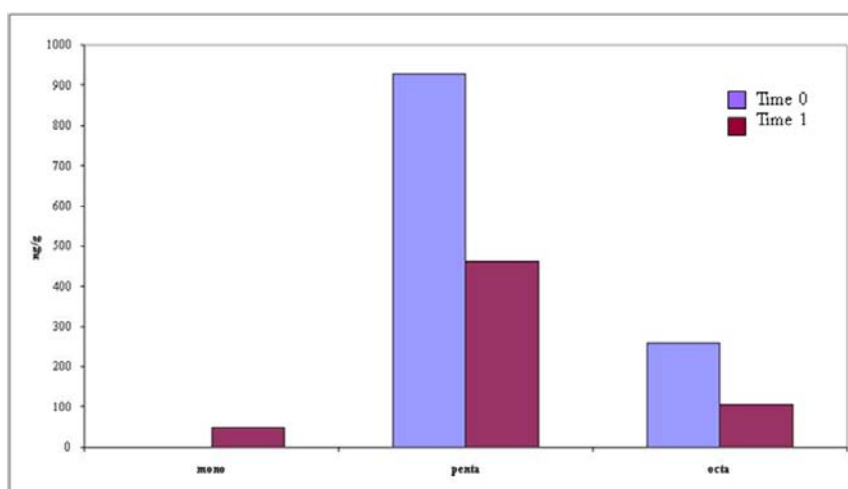


Figure 3. Penta, octa and deca-BDE mixture concentrations (ng/g) in activated sludge of the bench scale pilot plant before (time 0) e after (time 1) microbial enrichment.

4 CONCLUSIONS

All primary sludge samples of the 8 waste water treatment plants (WWTPs) tested in North (5) and Centre (3) Italy showed the presence of deca-BDE and mono, penta e octa-BDE mixtures. Elevated levels of deca-BDE were found, and this congener were between 73% and 99% of total BDE.

Results of this study showed also that microbial PBDEs degradation occurred in the

experiments on bench scale pilot plant, with a markedly decrease of the BDE with eight bromines or less after enriched in the activated sludge with two PCB-degrading bacteria (*Burkholderia xenovorans* and *Rhodococcus jostii*): halving penta-BDE mixture, great reduction of octa-BDE mixture.

These preliminary data indicate that these PBDEs can be aerobically transformed by PCB-degrading bacteria and this could have potential implications for the environment and public health.

Acknowledgments

This project was partially funded by the Italian Ministry of Education, University and Research (MIUR), through the program “Research Program of Relevant National Interest”, PRIN 2007PM3TJM_002 (Project title: *Water bodies protection from xenobiotic compounds: new methods for the analysis, the control and the treatment in civil and industrial wastewaters*).

REFERENCES

- Bedard, D.L., R Unterman, L.H., Bopp, M.J., Brennan, M.L. and Haberl, C.J. (1986). Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. *Appl. Environ. Microbiol.* 51: 761–768.
- Birnbaum, L.S. and Cohen Hubal, E.A. (2006) Polybrominated diphenyl ethers. A case study for using biomonitoring data to address risk assessment questions. *Environmental Health Perspectives* 114 (119): 1770-1775.
- Denef, V.J., Park, J., Tsoi, T.V., Rouillard, J.M., Zhang, H., Wibbenmeyer, J.A., Verstraete, W., and de Wit, C.A. (2002) An overview of brominated flame retardants in the environment. *Chemosphere* 46: 583-624.
- Denef, V.J., Park, J. Tsoi, T.V., Rouillard, J.M., Zhang, H., Wibbenmeyer, J.A., Verstraete, W., Gulari, W., Hashsham, S.A. and Tiedje, J.M. (2004). Biphenyl and benzoate metabolism in a genomic context: Outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. *Appl. Environ. Microbiol.* 70: 4961-4970.
- Dye, J.A., Venier, M., Zhu, L., Ward, C.R., Hites, R. A. and Birnbaum, L.S. (2007) Elevated PBDE levels in pet cats: sentinels for humans? *Environ. Sci. Technol.* 41: 6350-6356.
- FAO/WHO. Joint FAO/WHO expert committee on food additives (JECFA). Sixty-fourth meeting. Rome, 8-17 February 2005. Web site: http://www.who.int/ipcs/food/jecfa/summaries/summary_report_64_final.pdf
- Gerecke, C.A., Hartmann, P.C., Heeb, N.V., Kohler, H.P.E., Giger, W., Schmid, P., Zennegg, M. and Kohler, M. (2005) Anaerobic degradation of decabromodiphenyl ether. *Environ. Sci. Technol.* 39 (4): 1078-1083.
- Goris, J., De Vos, P., Caballero-Mellado, J., Park, J.H., Falsen, E., Tiedje, J.M. and Vandamme, P. (2004) Classification of the PCB- and biphenyl degrading strain LB400 and relatives as *Burkholderia xenovorans* sp. nov. *Intl J. Syst Evol Microbiol.* 54: 1677-1681.
- Gulari, S., Hashsham, A. and Tiedje, J.M. (2004). Biphenyl and benzoate metabolism in a genomic context: Outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. *Appl. Environ. Microbiol.* 70: 4961-4970.
- He, J., Robrock, K.R. and Alvarez-Cohen, L. (2006) Microbial reductive debromination of polybrominated diphenyl ethers (PBDEs) *Environ. Sci. Technol.* 40: 4429-4434.
- La Guardia, M.J., Hale, R.C. and Harvey E. (2007) Evidence of debromination of decabromodiphenyl ether (BDE-209) in biota from a wastewater receiving stream. *Environ. Sci. Technol.* 41 (19): 6663-6670.

- Law, R.J., Allchin, C.R., de Boer, J., Covaci, A., Herzke, D., Lepom, P., Morris, S., Tronczynski J. and de Wit, C.A. (2006) Levels and trends of brominated flame retardants in the european environment. *Chemosphere* 64:187-208.
- Martin, K.M., Rossing, M.A., Ryland, L.M., DiGiacomo, R.F. and Freitag, W.A. (2000) Evaluation of dietary and environmental risk factors for hyperthyroidism in cats. *J. Am. Vet. Med. Assoc.* 217: 853-856.
- Nyholm, N., Ingerslev, F., Berg, U.T., Pedersen, J.P., Frimer-Larsen, H. (1996) Estimation of kinetic rate constants for biodegradation of chemicals in activated sludge wastewater treatment plants using short term batch experiments and $\mu\text{g/L}$ range spiked concentrations. *Chemosphere* 33 (5): 851-864.
- Robrock, K.R., Coelhan, M., Sedlak, D.L. and Alvarez-Cohen, L. (2009) Aerobic biotransformation of polybrominated diphenyl ethers (PBDEs) by bacterial isolates. *Environ. Sci. Technol.* 43: 5705-5711.
- Sharp, J.O., Wood, T.K. and Alvarez-Cohen, L. (2005) Aerobic biodegradation of N-nitrosodimethylamine (NDMA) by axenic bacterial strains. *Biotechnol. Bioeng.* 89 (5): 608-618.
- Schecter, A., Papke, O., Tung, K.C., Staskal, D. and Birnbaum, L. (2004) Polybrominated diphenyl ethers contamination of United States food. *Environ. Sci. Technol.* 38 (20): 5306-5311.
- Schecter, A., Papke, O., Tung, K.C., Harris, J.J. and Dahlgren, J. (2005) Polybrominated diphenyl ethers flame retardants in the U.S. population: current levels, temporal trends, and comparison with dioxins, dibenzofurans, and polychlorinated biphenyls. *J. Occup. Environ. Med.* 47 (3): 199-211.
- Seto, M., Kimbara, K., Shimura, M., Hatta, T., Fukuda, M. and Yano, K. (1995) A novel transformation of polychlorinated biphenyls by *Rhodococcus* sp. strain RHA1. *Appl. Environ. Microbiol.* 61 (9): 3353-3358.
- Zota, A.R., Rudel R.A., Morello-Frosch, R.A. and Brody J.G. (2008) Elevated house dust and serum concentrations of PBDE in California: unintended consequences of furniture flammability standards? *Environ. Sci. Technol.* 42 (21): 8158-8164.