Analysis of PCDDs/PCBs by AhR-CALUX assay in Indian dietary food and environment ash

Masafumi Nakamura
Hiyoshi Corporation
Shunkei Ko
Hiyoshi Corporation
Muthusamy Govarthanan
Kyungpook National University
Vimalkumar Krishnamoorthi (Vimalkumar.Krishnamoorthi@nyulangone.org)
NYU Langone Medical Center: NYU Langone Health https://orcid.org/0000-0002-2278-1480

Research Article

Keywords: PCDD/Fs, DL-PCBs, Bovine milk, Municipal dump ash, CALUX Assay

Posted Date: October 20th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-356344/v2

Abstract

This study found distribution of polychlorinated dibenzo-*p*-dioxins/polychlorinated dibenzofurans (PCDD/Fs) and dioxin like polychlorinated biphenyls (DL-PCBs) in bovine milk and ash samples collected from 9 different major districts namely Erode, Salem, Namakkal, Coimbatore, Trippur, Madurai, Chennai, Dindigul and Tiruchirappalli, which were highly populated and industrialized districts of South India (Tamil Nadu). The total toxic equivalency (TEQ) for PCDD/Fs and DL-PCBs in the bovine milk samples were ranged from 0.028 to 7.331-pg TEQ/g fat. Some of the districts showed higher PCDD/Fs and DL-PCBs concentration in milk and ash samples. Further, BM14 (7.331 pg-TEQ/g fat) and BM21 (6.406 pg-TEQ/g fat) sampling sites showed exceed level of PCDD/Fs and DL-PCBs than WHO regulation limits (6 pg-TEQ/g fat). Similarly, Total dioxins and DL-PCBs concentration in the ash sample were between 0.003-1. ng TEQ/g. Samples from AS3 (1.2 ng-TEQ/g) and AS11 (1.06 ng-TEQ/g) showed higher total dioxins and DL-PCBs level among other sampling sites. This study provides an overview of dioxins and dioxin-related compounds contamination in bovine milk and ash samples in south Tamil Nadu. Further, the CALUX assay method validation has simplified the monitoring of dioxin contamination in the environment.

Introduction

Several studies reported most of the halogenated chemicals as persistent organic pollutants (POPs) in the environment. It is because of continuous uses and finally released into the environment. Most of the organic pollutants are harmful to human while it can be reach through air, water, and food products. The polychlorinated dibenzo-*p*-dioxins/ polychlorinated dibenzofurans (PCDDs/PCDFs) are also known as endocrine disrupting compounds (EDCs); consist of 75 PCDDs and 135 PCDFs. Similarly, polychlorinated biphenyls (PCBs) are a group of 12 PCBs with similar chemical and toxicological properties of PCDD/Fs. Dioxin like PCBs (DL-PCBs) are carcinogenic and included in group I (Lauby-Secretan et al., 2013). Both of them are chemically inert and resistant to biodegradation, hence persistent in the environment for long period (M Esposito et al., 2010).

PCDD/Fs and PCBs are highly lipophilic in nature and can easily accumulate in the fat tissues and finally bio – magnify to higher tropical organism. This will lead to severe effects on animals as well as humans and cause adverse effects on environment. Primary source of these PCDD/Fs and PCBs are industrial sectors like metallurgical production, paper production, pesticide manufacturing, etc. (Esposito et al., 2009; M Esposito et al., 2010). Emission of PCDD/Fs and DL-PCBs from the industrial sectors ultimately contaminates the dietary sources. For example, bovine milk from Northern Italy was highly contaminated by dioxins and PCBs, due to industrialization and agricultural activities (Bertocchi, Ghidini, Fedrizzi, & Lorenzi, 2015). Humans are mainly exposed through diet, almost 90% of human exposure via food. Some literatures illustrate that, milk and dairy products are significant sources for PCBs and dioxins (27.5-49.6%) in humans (Authority, 2012). A higher concentration of dioxins and PCBs had observed in animal origin foodstuffs like milk, meat, fish, and dairy products (Bocio & Domingo, 2005; Kiviranta, Ovaskainen, & Vartiainen, 2004). This becomes a significant concern among researchers to monitor the dioxins and

DL-PCBs in environment. Many techniques are available to measure PCDD/Fs and DL-PCBs concentration in environmental matrices (Croes et al., 2013; García-Bermejo et al., 2015; Lv, Zheng, Liu, Liu, & Xiao, 2011; Subedi & Usenko, 2012).

The conventional methods (high- resolution mass spectrometry/high- resolution gas chromatography (HRMS/HRGC)) are applied to measure dioxins in different samples (Kanan & Samara, 2018). This method is high accurate and outstanding reliability of separate the specific chemicals. HRMS/HRGC takes approximately 4-6 weeks to obtain the concentration of dioxin like compounds in different environmental matrices. The toxic equivalency (TEQs) obtained by HRGC/HRMS was compared with the mixture of known toxic equivalency factor (TEF) compounds. The most common toxic congener 2,3,7,8-TCDD was used to obtain the TEQ of unknown compound. However, some of the samples cannot be store for long time like pasteurized milk, because milk can only store for approximately 7-10 days at 4-7 ºC.

The chemically activated luciferase gene expression (CALUX) bioassay is used to obtain the concentration of PCDD/Fs and DL-PCBS in samples in short period (3-7 days). Further, this assay directly provides the total sum of toxic equivalency (TEQ) of tested samples instead of providing information of which congener contributes to the total PCDD/Fs concentration. Many scientists determined the PCDD/Fs in different samples including marine organisms (eels, mussels, and fish), food stuffs (meat and meat products (cod liver, hot dogs, hamburger, chicken breast, lamb chop), vegetables (tomato, lettuce, spinach, green beans), fruits (apple, orange, banana), cereals, egg, oils and fat (coconut oil, olive oil, sunflower oil, corn oil and margarine), pulses and dairy products (cheese, yogurt, cream caramel, custard)), feed, human tissues (blood plasma and human milk), and environmental matrices (soil and sediment). An another study reported the concentration of PCDD/Fs in cow's milk (Bocio & Domingo, 2005; Godliauskienė, Tamošiūnas, & Naujalis, 2017; González, Marquès, Nadal, & Domingo, 2018; Kanan & Samara, 2018; Lake et al., 2013; Lorenzi et al., 2020)

The mechanism that involved in the CALUX was an important assay to determine the concentration of PCDD/Fs and DL-PCBs in milk samples. Dioxins and related chemicals cause effects on the cells through the aryl hydrocarbon receptor, which is a transcription factor. Activation of aryl hydrocarbon receptor (AhR) by dioxins modifies the gene expressions, involved in the cell growth and differentiation (Cancer, 1997). Alterations in the gene expression will leads to various toxic effects. The concentration of dioxins and dioxin like compounds in different samples had quantified by CALUX assay. This is an *in vitro* bio-analytical tool applied in research and commercial laboratories. CALUX assay is a reporter gene-based cell bioassay; the genetically modified cells used for this assay. The cells that contain the luciferase reporter gene, which response to the dioxin and dioxin-like chemicals. This gene expression was induced by binding and activating of the AhR. The luciferase gene expression depended on the time, dose, AhR dependent and chemical specific manner. The chemical concentration in the environmental samples was measured by the amount of luciferase enzyme produced by binding and activation of AhR. The

luminescence by chemical was converted to bioassay TEQ value by comparing the response against the given dose of chemical and response to 2, 3, 7, 8- tetrachlorodibenzo-*p*-dioxin (TCDD). CALUX assay was used to analyze all AhR active compounds in the extract samples. However, the result interpretation could be complicated compared to chemical analysis because the extracted sample contains a mixture of chemicals (Han, Nagy, & Denison, 2004). Presence of other contaminants (metals) could affect the CALUX assay readings. Metals in the samples can be able to inhibit the transcription of AhR gene. AhR was associated with the Hsp90 in the cytoplasm, which translocate to the nucleus while it strong affinity with ligands (contaminants or metal). Hsp90 was switched off from the AhR and make a heterodimer bond with AhR nuclear translocator (Arnt). This complex was binds on the xenobiotic response element (XRE) located on the upstream of the CYP1A1. AhR and Arnt is comes under the bHLH transcription factor, another polypeptide sequence from the same family have the ability to repress the AhR activity known as AhR repressor (AhRR). This repressor competing with AhR and make a bond with Arnt and XRE sequence. Hence, AhR expression was regulated by the feedback inhibition of AhRR. Inhibition of AhR function ultimately results in the lack of luciferase enzyme production (Chao et al., 2006; Mimura, Ema, Sogawa, & Fujii-Kuriyama, 1999).

The objective of this study was 1) to determine the levels of PCDDs/PCBs in bovine milk and ash samples from various dumping sites 2) to understand the AhR-CALUX bioassay mechanisms and pathways 3) the distribution of PCDD/Fs and PCBs among sampling location in south Tamil Nadu.

Materials And Methods

2.1 Chemical reagents and solvents

Hiyoshi Corporation, Japan, provided facility and utilized following chemicals hexane (for pesticide residue analysis), RPMI 1640 media, 545 celite, D-PBS and Nonane (for dioxin analysis), Ethyl acetate (for pesticide residue analysis), Acetone, Toluene and sulfuric acid, Sodium sulfate and silica gel (for making column).

2.2 Sample collection

Bovine milk and municipal dump ash samples of urban and rural areas were collected (January – April 2012) from the different urban and rural regions (districts) of South India (Figure 1). Briefly, 25 milk samples (150 mL of Bovine Milk), and 16 ash samples (50 g of Ash sample) were collected in dumping sites. Milk samples were pack into the screw-type bottle and ash samples covered with zip lock covers.

2.3 Extraction of milk samples

80 mL of milk sample was mixed with 150 mL of acetone in the glass-separating funnel. Then the samples were extracted with 40 mL of hexane. The samples were settled, and the top layer passed through a 25 mL of glass extraction column. Sample and sodium sulfate mixture was packed in an extraction column filled with 20 mL of hexane and kept for 10 min. Then milk-hexane layer passed into the column. After washing the column, the total solvent has completely evaporated, and then sample vial has been measured for fat content.

2.4 Extraction of Ash sample

Two grams of ash sample was weighed and mixed with 20 mL of 2 N of HCl was added. After one hour, HCl-extract was washing with deionized water. The pH of the extract was checked and finally washed with methanol. The obtained HCl extract containing dioxin transferred into the sox let distillation unit. 250 mL of toluene was used for extraction for 16 h.

Later, 100 mL of Dichloromethane (DCM) was mixed with 10 mL of sample. The mixture was transferred into the liquid-liquid extraction column and extracted for 10 min for three times. Then the sample was condensed up to 20 mL and further processed. After completing the extraction, the ash sample transferred into 30 mL test tubes. The total 20 mL of ash sample extract added 4 mL into clean, dry test tubes, and then 20 µL of nonane was mixed with each ash samples, and then kept the samples in multi-solvent extraction unit for 20 mins.

2.5 CALUX Bioassay

The patented dioxin responsive mouse recombinant cell line, H1L6.1c2 was obtained from the Hiyoshi Corporation, Japan (Kumar & Segen, 2001; Sakthivel, Balasubramanian, Nakamura, Ko, & Chakraborty, 2016) and the method developed by Xenobiotic Detection System (XDS, USA). The cleaned extract to concentrated and then passed through two columns, first with 25 mL of acid silica column and second with 5 mL of active carbon column. 5 mL of the disposable column was packed with following materials, by glass wool, 0.3 cc of sodium sulfate, 0.7 cc of XCARB (1%), which was an activated carbon patented by XDS (USA) and 0.6 cc of sodium sulfate, rinsed with 5 mL acetone, 10 mL toluene and finally with 10 mL of Hexane. A 10 mL of the disposable column was filled from bottom to top, by glass wool, 1.0 cc sodium sulfate, 4.3 cc of 33% acid silica gel, and 1.0 cc of sodium sulfate and rinsed with 30 mL of Hexane. The acidic silica column placed on top of the carbon column. The fraction containing the Co-PCBs (PCB fraction) eluted with 15 mL ethyl acetate/toluene/hexane (1:1:8) and the second fraction containing PCDD/F (dioxin fraction) was eluted afterwards with 20 mL of toluene. Extracts were concentrated to dryness in a centrifuge under vacuum and reconstituted with 4 mL of Hexane. The samples were stored at 4 ºC until analysis.

Different concentration (lower to a higher level) of 2,3,7,8 – Tetrachloro dibenzo dioxin standard was prepared for the slope of calibrating the CALUX assay (9 point calibration) system (Sakthivel et al., 2016). The purified sample extracts in DMSO were suspended in a cell culture medium. Before dosing the plate, hexane solution was transferred into 4 µL of DMSO under vacuum condition in the centrifuge, and finally, 400 µL medium were added to each extract in DMSO. A 2, 3, 7, 8 TCDD standard curves were generated on each plate in addition to the samples. A mixed solution of PCDD/Fs was analyzed for quality control and DMSO alone was studied as blank. This is further serially diluted ranging from 100, 200 µL up to 900 µL and added to the 9 well plates (STD1 – STD9). After 20-24 h of incubation, cells were examined microscopically for obvious toxicity. Each well rinsed with 50 µL of phosphate- buffered saline (PBS), and 30 µL of cell culture lysis reagent were added to each well then. The plate was shaken for 2 min at room temperature before being placed in the luminometer. After luciferase assay reagent, the light output was integrated, and results were expressed in relative light unit (RLU).

3. Mechanism of CALUX Assay

The recombinant cell line used in this assay (H1L6.1c2) was generated by stably transecting the plasmid pGudLuc6.1. This plasmid contains the CYP1A1 dioxin responsive domain. PCDD/Fs and PCBs, bind to the AhR. The PCDD/Fs and PCBs-AhR complex then travels to the nucleus of the cell. Activated PCDD/Fs and PCBs-AhR then binds to specific sequences in the DNA called dioxin responsive elements (DRE).

The binding of the PCDD/Fs and PCBs-AhR complex to the DRE will undergo transcription and produce transcripts altered by the founded dioxin. The resulted transcript will direct to luciferase synthesis. The messenger RNA (mRNA) then transfers to the cytoplasm for translation (protein synthesis). New proteins will be synthesized from the polypeptides, called luciferase enzyme. The synthesized protein can cause toxic effects. Dioxin TEQ was measured from luminescence produced by the luciferase reporter gene (Figure 2). Addition of luciferin (substrate) results in the catabolism by luciferase enzyme, which produces the light measured by luminometer. The produced light was directly proportional to the evoked effects by PCDD/Fs and PCBs-AhR.

Results And Discussion

4.1 PCDD/Fs and DL-PCBs in bovine milk samples

Figure 3 describes the PCDD/Fs and DL-PCBs in bovine milk samples from major cities in Tamil Nadu were determined by CALUX bioassay. Total dioxin concentration was higher in BM14 (7.331 pg-TEQ/g fat) followed by BM21 (6.406 pg-TEQ/g fat), BM8 (4.473 pg-TEQ/g fat) and BM3 (4.237 pg-TEQ/g fat). The lowest concentration was found at BM13 (0.028 pg TEQ/g). Total dioxin like compounds concentration in bovine milk samples was given in Table 1. The plausible reason for higher concentration of dioxin like compounds in milk at BM14 and BM21 could be highly populated and industrialized

districts, which dispose large number of polymers (plastics), e-waste and other domestic wastes in the dumping sites. Cows around the dumping sites had been exposing to dioxin like compounds through inhalation, dermal adsorption, and ingestion. They not only ingesting the feeds, at the same time they fed polymers too. Burning of plastic wastes had increases the distribution of dioxins and DL-PCBs to the surrounding areas and leads to exposure of cows via skin. Synthetic polymers are major source for dioxin like compounds in environmental matrices and the associated organisms. Because plastics are economically in-expensive, light weight and their various properties captivating the peoples. Hence, plastics have been producing in large quantities as single use plastics, which results in the higher plastics in municipal dumping sites. According to Jambeck et al. (2015) disposal rate of plastics in 1960 was less than 1%, however this had been increasing to more than 10% by 2005 in developed and developing countries. The disposed plastics are not easily biodegrade in the environment, hence dumping of plastics wisely increasing the concentration of plastic associated chemicals in the environment (Barnes, Galgani, Thompson, & Barlaz, 2009).

The major source of dioxin exposure on humans had been consuming contaminated animal source foods, like milk, egg, meat, and fish. The European Union strictly regulated the presence of dioxin and DL-PCBs in food sources to avoid human exposure. In such case, the maximum level of dioxins and DL-PCBs in milk and dairy products was 6.0 pg TEQ/g fat. The maximum limit of PCDD/Fs in milk and milk products should be 3 pg TEQ/g fat. But the maximum value obtained in the present study was 7.331 pg-TEQ/g fat levels, and the overall average level was 3.027 pg-TEQ/g fat. PCDD/Fs and DL-PCBs obtained in the current study revealed that the dioxin levels were very high compared to the values of Chou et al. (2008). These values show an alarming situation to the farmers and consumers. Some literatures reported the presence of PCDD/Fs and DL-PCBs in raw milk at below to the maximum residues limit in milk established by EU. In such case, raw milk collected from 9 regions of South Korea showed PCDD/Fs and DL-PCBs concentration ranged between 0.05-0.56 pg TEQ/g fat (average concentration-0.27 pg TEQ/g fat) and 0.15-0.40 pg TEQ/g fat (average concentration-0.33 pg TEQ/g fat). In this, concentration of DL-PCBs had been higher than PCDD/Fs and PCDFs concentration was highly contribute to the total PCDD/Fs concentration than that of PCDDs in milk samples (Kim, Kim, Jang, Bong, & Kim, 2013). Further, PCDFs level in 2008 was considerably lower than reported in 2005. This may be due to the implementation and strong restrictions of emission from the incineration plants in 2003. Higher chlorinated PCDFs were emitted by the combustion of wastes than that of lower chlorinated PCDFs. Few more scientists reported similar pattern of PCDD/Fs due to waste combustion. Further, Combustion was the primary source of dioxin like compounds in food chain (Mauro Esposito et al., 2010).

The present study was compared to dioxins concentration in pasteurized milk. Toxic equivalency of PCDD/Fs for pasteurized milk sample by CALUX assay was ranged from 0.012 to 0.748 pg – TEQ/g fat levels and the overall average value was 0.246 pg TEQ/g fat (Chou et al., 2008). One more study reported the dioxins and DL-PCBs level in pasteurized bovine milk by Monte Carlo simulation modelling. The mean dioxin and DL-PCBs in pasteurized milk were 0.06 $\pm$ 0.07 pg WHO-TEQ/g (Adekunte, Tiwari, & O'Donnell, 2010). Many literatures showed consumption of milk and dairy products had attributed the

dioxin and DL-PCBs exposure on humans. About 38% of the Dutch populations were affected by dioxins and DL-PCBs by consuming contaminated milk and milk products (Sapkota, Lefferts, McKenzie, & Walker, 2007). The reason for dioxins and DL-PCBs in milk could be the feed that cow consuming, which influences on the exceeding level of dioxin and DL-PCBs in milk than the EU-regulated level. R. Hoogenboom et al. (2010) reported that cows feed (potato peels had kaolinic clay) containing high level of dioxins resulted in the huge milk contamination. One study reveals that higher dioxins and DL-PCBs were observed in milk, due to grazing of the cow on contaminated pastures near to factories that produced PCBs (Turrio-Baldassarri et al., 2009). In some cases, consumption of roughage by cow could leads to the presence of dioxins and DL-PCBs in milk because roughage was the predominant feed source for dairy cows. Further, ingestion of contaminated soil during grazing was also lead to dioxins contamination in milk (Rychen, Jurjanz, Toussaint, & Feidt, 2008). Lactation period of cow helps to eliminate the dioxins and DL-PCBs through milk. Early lactation period of cow results in higher elimination of dioxins and DL-PCBs in milk fat because cow utilizes the stored fat to balance their energy level. Additionally, dioxins and DL- PCBs are lipophilic; hence, they accumulate in the body fat. Early lactation period will release the accumulated dioxins because the cows are in negative energy balance (Brambilla et al., 2008).

Milk and dairy products from official control program carried out in Belgium showed a higher level of PCDD/Fs and DL-PCBs determined by HRMS/HRGC; the reported concentration was ranged from 1.58 to 1140.35 pg bio-analytical equivalents (BEQ)/g fat (mean: 31.36 pg BEQ/g fat), respectively (Vromman et al., 2012). Similarly, few more literatures reported the mean concentration of dioxins and DL-PCBs in milk from different countries, like Italy (0.71 pg WHO TEQ/g; DL-PCBs: 1.39 pg WHO TEQ/g) (Esposito et al., 2009; Fattore, Fanelli, Turrini, & Di Domenico, 2006), China (0.03 pg WHO TEQ/g) (J.-G. Li, Wu, Zhang, & Zhao, 2007), and Belgium (1.57 + 0.71 pg WHO TEQ/g; DL-PCBs: 1.13 pg WHO TEQ/g) (Bilau et al., 2008; Windal et al., 2005). Milk samples from farms located in Brescia City were used to determine PCDD/Fs and DL-PCBs contamination level by HRMS, and the total WHO-TEQ values were ranged from 1.78 to8.16 pg WHO-TEQ/g fat (average: 4.13 pg WHO-TEQ/g fat), respectively. Higher DL-PCBs in the milk may be due to the steel industry, landfills and waste-energy plant and Caffaro factory (produced a higher volume of PCBs) vicinity to study area (Bertocchi et al., 2015). On the other hand, PCDD/Fs and DL-PCBs in milk had been measured from 115 milk-collecting plants in France. The quantified concentration rage of PCDD/Fs was 0.16- 0.76 pg TEQ/g fat (median: 0.47 pg TEQ/g fat) and DL-PCBs concentration was varied between 0.35 – 1.35 pg TEQ/g fat (median: 0.47 pg TEQ/g fat), respectively. The total concentration of dioxins and DL-PCBs were ranged from 0.60 to 1.77-pg TEQ/g fat (0.78 pg TEQ/g fat). DL-PCBs contribute about 62% to the total TEQ. Consumption of contaminated corn by dairy cows results in a higher level of dioxins and DL-PCBs in milk samples. The source for dioxins could be incineration of waste materials by scrap merchant (Durand et al., 2008). The above-mentioned sources of PCDD/Fs and DL-PCBs corroborate with the present study and illustrated the association between dioxins concentration in the milk and the sampling area. Incineration of waste, grazing of cow by polluted area could be the plausible reason for higher PCDD/Fs and DL-PCBs in milk. PCDD/Fs concentration was significantly higher than that of DL-PCBs in milk samples from most of the sampling sites.

To assess the source of dioxins and PCBs in the milk samples, R. L. Hoogenboom et al. (2015) fed the dairy cows with maize silage that contaminated by the fire in the barn using large amount of polyvinyl chloride (PVC) plastics (smoke that enter into the maize field) and sugar beet pulp (SBP) contaminated by the broken plastic roof in the coal used for drying. The authors and co-workers determined PCDD/Fs and DL-PCBs in the milk by GC/HRMS and stated that these dioxins levels were dependent on the feed and background exposure. These feds were given for 33 days alternatively with other feed ingredients for nutritional requirement. PCDD/Fs and DL-PCBs level had been higher in the cow milk fed with maize silage (2.6 pg TEG/g fat and 1.0 pg TEG/g fat) compared to SBP (1.7 pg TEG/g fat and 0.5 pg TEG/g fat). On the other hand, among these two compounds, concentration of PCDD/Fs was higher than that of DL-PCBs. Carry over rate of PCDD/Fs from fed to milk was higher for maize silage (25%), but DL-PCBs carry over rate was higher for SBP (35%). On the contrary, Slobs, Olling, Derks, and De Jong (1995) stated that carry over rate of PCDD/Fs from the fly ash to milk was slower. Further, contaminated fed and grazing of cow by municipal waste incinerators not greatly influence on the PCDD/Fs level in milk. Whereas Schuler et al. (1997) reported the higher carry over rate of dioxin like compounds via sampling site near to modern waste municipal incinerator. Hence, burning of polymers and presence of plastics in the animal fed influence on the higher PCDD/Fs and DL-PCBs in milk. Moreover, incineration of plastic wastes affects the surrounding distance of 1 km (L. Hoogenboom, Hoffer, Mennen, Morgenstern, & Traag, 2012). In such scenario, milk samples taken by dumping sites ultimately influence on the higher exposure to dioxin like compounds.

Bovine milk from different countries showed the shift in contaminatioFig.2n level of milk by various sources. Contamination level of PCDD/Fs and DL-PCBs in cow's milk from Austria was 0.14 pg TEQ/g fat and 0.83 pg TEQ/g fat; similarly, milk from German showed PCDD/Fs and DL-PCBs concentration of 0.45 pg TEQ/g fat and 0.97 pg TEQ/g fat) (Thanner & Moche, 2004). Schmid, Gujer, Zennegg, and Studer (2003) reported the variation in dioxins and DL-PCBs contamination level based on origins, like a farm (a point source) (0.63$\pm$0.26 pg TEQ/g fat) and remote areas (0.36$\pm$0.09 pg TEQ/g fat). Milk samples from 63 farms in Italy were collected by personnel of Veterinary Authorities of Regional Sanitary Service (AASSLL) and determined the dioxins and DL-PCBs concentration by HRMS. Total PCDD/Fs and DL-PCBs concentration was ranged between 0.46 and 26.80 pg/g milk fat (mean 3.06 pg/g milk fat). Some of the milk samples were found exceed dioxins contamination (above EU regulation limit), close to Napoli and Caserta districts, which contaminate the buffalo herd. However, Dioxin and DL-PCBs concentration did not exceed the EU regulation limit in the remaining 58 samples (Esposito et al., 2009). Another study also found bovine milk contamination collected from 50 farms in risk areas in 2008-2014 (Italy). The obtained result for dioxins and DL-PCBs ranged as 0.59-2.36 WHO-TEQ pg/g fat, 0.70-1.14 WHO-TEQ pg/g fat and 0.25-2.75 WHO-TEQ pg/g fat for buffalo, bovine and sheep milk, respectively (Serpe, Scaramuzzo, Maglio, Lambiase, & Esposito, 2015).

The concentration of dioxins and DL-PCBs in bovine and ovine milk was determined by HRMS. The sampling areas were close to incineration plant (Valpiana area and Casone area) in Tuscany, which leads to PCDD/Fs and DL-PCBs in milk samples ranged from 0.71 to 2.9 pg WHO-TEQ/g fat (Ingelido et al., 2009). However, the concentration range was not exceeded the EU regulation (PCDD/Fs: 3 pg WHO-

TEQ/g fat and Total TEQs: 6 pg WHO-TEQ/g fat) and action levels (PCDD/Fs and DL-PCBs: 2 pg WHO-TEQ/g fat), respectively (Commission, 2006). The incineration plants were processing municipal solid wastes in large amount, which was the primary source for dioxins contamination to the surrounding area. Most of the literatures described waste incineration, in specific polymer wastes could imply on the higher PCDD/Fs and DL-PCBs in milk samples, further in the present study the determined dioxin like compounds concentration had been higher than the EU regulation limit. This may lead to adverse human health effects who are consuming the contaminated milk.

4.2 PCDD/Fs and DL-PCBs in ash samples

Concentration of dioxin like compounds in ash samples from highly polluted districts in Tamil Nadu were given in Table 2. The concentration of PCDD/Fs and DL-PCBs in ash samples were ranged 0.0129 – 1.208 ng TEQ/g. Total dioxins and DL-PCBs concentration were higher at AS3 sampling site (1.208 ng TEQ/g) and AS11 (1.0675 ng TEQ/g) among other sampling locations. In specific, PCDD/Fs concentration had been higher in the AS3 and DL-PCBs in AS1 (0.044 ng TEQ/g). The dioxins concentration was higher in almost all the sampling sites than compared to DL-PCBs concentration. According to WHO values, the results infer that concentration of PCDD/Fs and DL-PCBs were under safety levels (Figure 2). Incineration of many organic materials under less-than-optimal conditions leads to release of higher dioxins and related compounds. In such case, the higher level of dioxins in ash samples from AS3 and AS11 illustrate the huge combustion of organic materials. Municipal solid waste incineration could be the point sources to release PCDD, PCDF and other toxicants; exposed by humans and other organisms (Davoli et al., 2010; Mari, Nadal, Schuhmacher, & Domingo, 2009; Yang, Park, & Lee, 2006). Particle size also influences on the dioxins concentration, fly ash generated from the municipal solid waste from major countries showed the dioxins concentration ranged from 0.47 to 25.74 ng I-TEQ/g. Fine ash particles had a higher level of dioxins than larger ash particles (S. Wu et al., 2016). Fly ash sample collected from sixteen municipal solid waste incinerators (MSWIs) in Taiwan results in 0.78-2.87 ng I-TEQ/g (average: 1.87 ng I-TEQ/g) of dioxins. Further, higher chlorinated PCDDs were highly contributing than lower chlorinated PCDDs (Y.-M. Chang et al., 2011). Another study reported the emission of PCDD/Fs and DL-PCBs by co-combustion of rice husk and polyethylene (PE) in the 4 municipal waste thermal power plant in Italy. The authors stated that combustion of plastic with biomass materials reduce the release of organic pollutants into the environment. PCDD/Fs and DL-PCBs were determined in fly and bottom ash by HRMS/HRGC. Rice husk replaced with 15% PE showed higher level of PCDD/Fs and DL-PCBs in fly ash than bottom ash. Composition of the ashes is dependent on the fuel properties and fly ashes were found higher concentration of PCDD/Fs and DL-PCBs. Further, PE was used as a fuel result in higher level of organic pollutants in the fly ash than found in bottom ash, because fly ashes had more adsorption area for pollutants (Colapicchioni et al., 2020; Masto et al., 2015).

Similarly, Cobo, Gálvez, Conesa, and de Correa (2009) reported dioxins and DL-PCBs in fly ash samples collected from incineration plant, which was quantified by HRGC coupled to ion trap low-resolution mass

spectrometry (QITMS/MS), which is used to processing medical (blood, tissues, body parts, and contaminated sharps) and industrial wastes (polymerization sludge, expired food products, and commercial products). Higher PCDD/Fs (>185 ng WHO-TEQ/g) and lower DL-PCBs (1.2 ng WHO-EQ/g) concentration were found in the bag filter ash samples. This was corroborating with ESP ash samples (142.1 ng I-TEQ/g) collected from old and small- scale incinerator. Higher PCDD/Fs concentration in fly ash may occur due to old and inefficient operation of wastes (batch process, outdated furnace, slow gas cooling system). Dioxins level in the ash samples were varies dependent on the type of waste materials that was incinerated. Different types of ash samples were monitored for dioxins level, in such case types of ash samples from MSWIs showed a different pattern of dioxin concentrations, such as bag filter ash (1.12 and 12.2 ng I-EQ/g), gas scrubber ash (0.26 ng I-TEQ/g), electrostatic precipitators (ESP) (6.7, 8.5 and 142.1 ng I-TEQ/g) and the mixture of semi-dry and bag filter ash samples (0.97-1.5 ng I-EQ/g) (He, Zhang, Zhang, & Lee, 2004; Yasuhara & Katami, 2007). Dioxin concentration in fly ash may vary depending on the specific operating parameters, like furnace type, capacity, furnace temperature and type of waste (M.-B. Chang & Chung, 1998). Pan et al. (2013) reported the dioxins and DL-PCBs concentration in fly ash collected from 15 municipal concrete waste incinerator plants in different cities of China. The concentration of PCDD/Fs and DL-PCBs in fly ash samples were ranged from 0.034 to 2.5 ng- TEQ/g, respectively. This was comparatively lower than dioxins level reported in Taiwan (780- 2860 ng TEQ/kg) (Y.-M. Chang et al., 2011), Zhejiang (China) (140-2680 ng TEQ/kg) (Chen et al., 2008), and 4 province in (Henan Province, Anhui Province, Jilin Province, Shandong Province and Hubei Province) China (0.0707-0.7742 ng I-TEQ/g) (Sun et al., 2017). The seasonal variation of dioxin distribution was studied by Sun et al., who discovered the increased dioxins concentration in Hubei Province during winter. However, seasonal variation was not an important factor influence on the PCDD/Fs in fly ash. Solidification of fly ash samples had been reducing the concentration of PCDD/Fs, and higher temperature in the thermal process leads to formation of PCDD/Fs (250 ºC). The TEQ values lower than 1.0 illustrates the lower toxic effects of PCDD than the PCDFs released from the municipal solid waste incinerators. Few more studies also found a higher level of dioxins than the concentration quantified in the present study. Those had showed dioxins level in fly ash from MSWIs in Shanghai, China (7.53, 1.52, 0.98-1.5, 4.16 ng-TEQ/g (Shanghai) and 0.005-87 ng I-TEQ/g (He et al., 2004; Jin et al., 2003; Liu, Jiang, Wang, Dong, & Zheng, 2015). The PCDD/Fs and DL-PCBs concentration was varied depending upon the particle size of the ash. Ash samples collected from MSWIs in China showed PCDD, PCDF and DL-PCBs concentration ranged from 0.19-2.27 ng I-TEQ/g, 0.2-4.9 ng I-TEQ/g and 0.02-0.22 ng I-TEQ/g, respectively. Dioxins concentration was higher in fine ash particles (diameter 10-2.5 and <2.5) than in the bulk ash (S. Wu et al., 2016). MSWI in southern Taiwan was studied to observe the dioxins and DL-PCBs level in fly ash. The reported dioxins concentration was ranged from 1.97×10^{-3} to 8.93×10^{-1} ng I-TEQ/g (median: 4.52×10^{-2} ng I-TEQ/g) (Hsieh, Chen, Zhu, Wu, & Huang, 2018), this was corroborated with other countries including Taiwan (laboratory waste: 0.02-1.86 ng I-TEQ/g), and Northeast China (fly ash from MSWI: 3.2-800.1 ng I-TEQ/g), respectively (J. Li et al., 2016; J.-L. Wu et al., 2014). However, these findings were relatively higher than those found at AS3 in the present study. Concentration of PCDD/Fs in the fly ash was changed on the daily basis, due to the instability of the incineration plants. Few more studies reported the

concentration of dioxins in fly ash from MSWIs in China (34-2500 ng WHO-TEQ/g) (Liu et al., 2015; Pan et al., 2013).

There was no positive correlation between PCDD/Fs and DL-PCBs in the bovine milk and ash samples from the major districts in Tamil Nadu, India. However, higher level of PCDD/Fs and DL-PCBs in both the samples was found higher in Erode, Dindigul, Salem, Madurai, and Chennai. The order of dioxin like compounds in bovine milk and ash sample from districts were, Coimbatore (BM14) > Chennai (BM21) > Salem (BM8) > Erode (BM3) > Dindigul (BM24) > Madurai (BM20) and Erode (AS3) > Dindigul (AS11) > Madurai (AS14) > Nammakkal (AS6) > Chennai (AS9) > Salem (AS1). Different source of PCDD/Fs and DL-PCBs were clearly described to find the reason for human exposure. This would help to understand the health effects of dioxins and DL-PCBs while exposed frequently.

Conclusion

In the present study, PCDD/Fs and DL-PCBs were determined from the bovine milk and ash samples collected from the municipal dumping sites in the major districts in Tamil Nadu by CALUX assay. Higher PCDD/Fs and DL-PCBs were found in bovine samples from BM14 followed by BM21, BM8 and BM3. Similarly, dioxin like compounds in the ash samples was higher at AS3 followed by AS11, AS14 andAS6. PCDD/Fs concentration was higher in almost all the sampling sites compared to DL-PCBs in bovine milk and the similar pattern was observed for ash samples. But there was no positive correlation between bovine milk samples and ash samples. The major source of PCDD/Fs and DL-PCBs was combustion of polymers in the municipal dumping sites.

Distribution of dioxin like compounds from one place to another by the fly ash leads to more toxicological effects on humans as well as other organisms. Size of the fly ash decides the composition of organic pollutants. The CALUX assay helps to directly get the TEQs of organic pollutants. PCDD/Fs and DL-PCBs in the bovine milk were higher than the EU regulation. Therefore, it should be of concern on the consumers in the highly polluted districts in the south Tamil Nadu. However, values of PCDD/Fs and DL-PCBs in ash samples were lower than the EU regulation. In developing countries as India had no proper solid waste management, hence it results in higher emission of toxic pollutants and the distribution rate is also higher to the nearby places. This study only provides the baseline data for the dioxin like compounds toxicity, future research is needed to understand the exposure of these pollutants to next generation by fetus and milk.

Declarations

Acknowledgement

The authors sincerely thank to HIYOSHI Corporation (Japan), to provide facilities and support for the entire research work. A special thanks to Dr. R. Babu Rajendran (Department of Environmental Biotechnology, Bharathidasan University) providing this opportunity to accomplish the work in Japan.

Declarations

Author contribution

Murugasamy Mayilsamy: investigation, data curation, validation and writing - original draft, **Seethappan Sangeetha:** investigation, data curation, validation and review, **Masafumi Nakamura**: method validation and investigation, **Shunkei Ko:** methodology, visualization and investigation, **Muthusamy Govarthanan**: visualization, investigation and formal analysis, **Krishnamoorthi Vimalkumar**: writing – review, edit, method validation and supervision.

Funding

This full work carried out and funded by Hiyoshi Corporation, Omihachiman, Shiga prefecture, Japan.

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Competing interests: The authors declare no competing interests.

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Figures

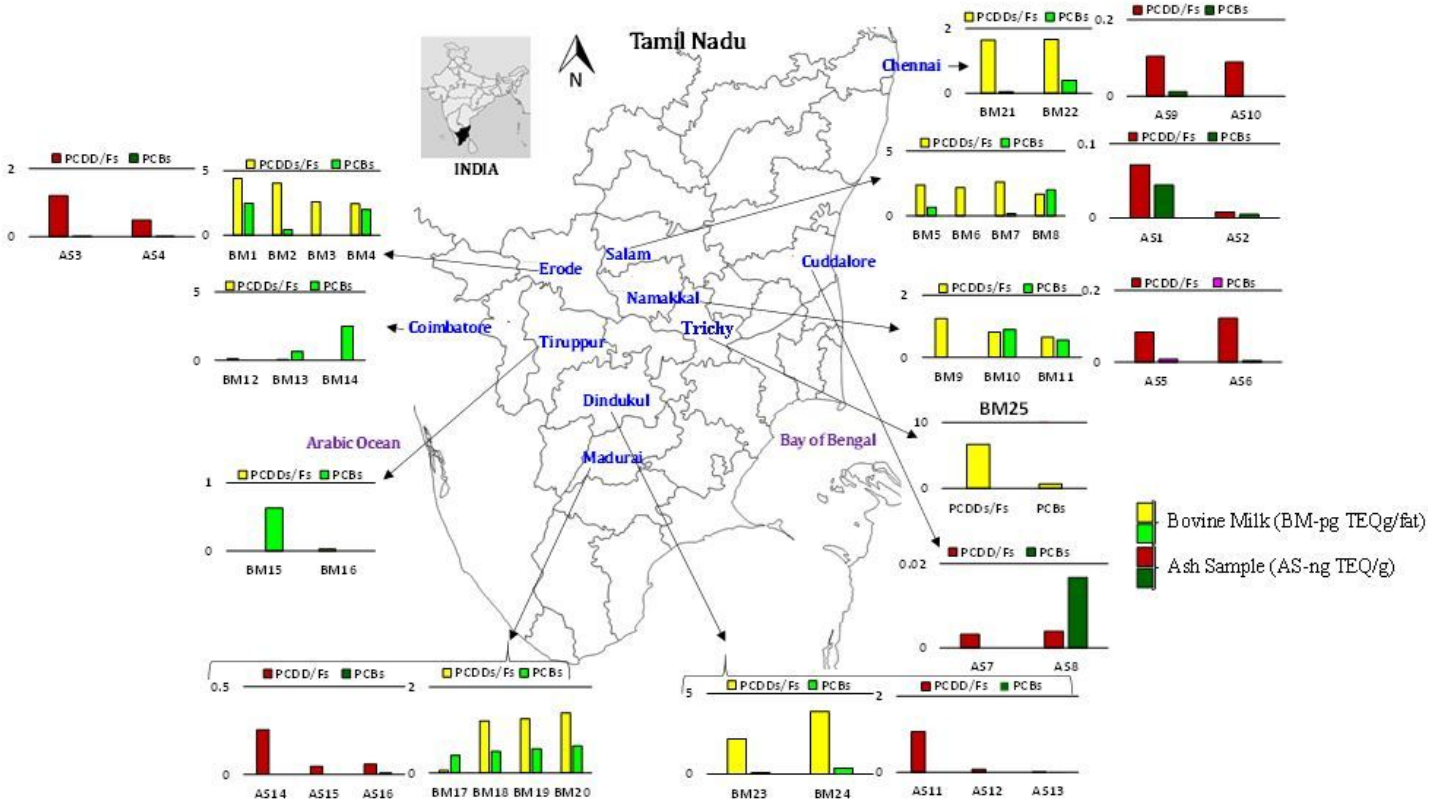

Figure 1

Bovine milk and Ash samples location of south Indian region

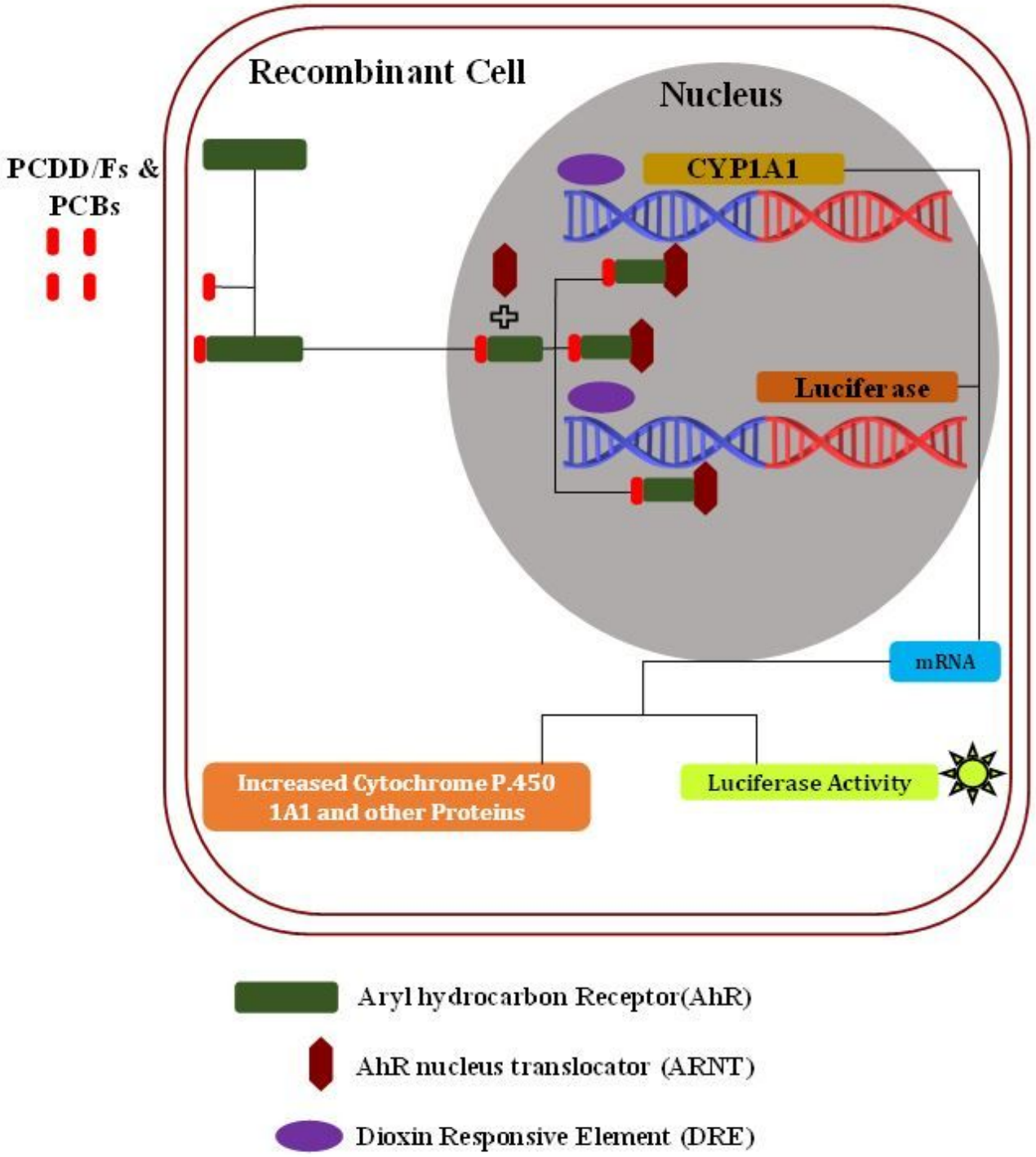

Figure 2

Schematic diagram represents the mechanism of CALUX Assay (Hiyoshi Corporation, Japan), AhR – Aryl hydrocarbon Receptor, ARNT – AhR Nuclear Translocator protein, DRE – Dioxin Responsive Element

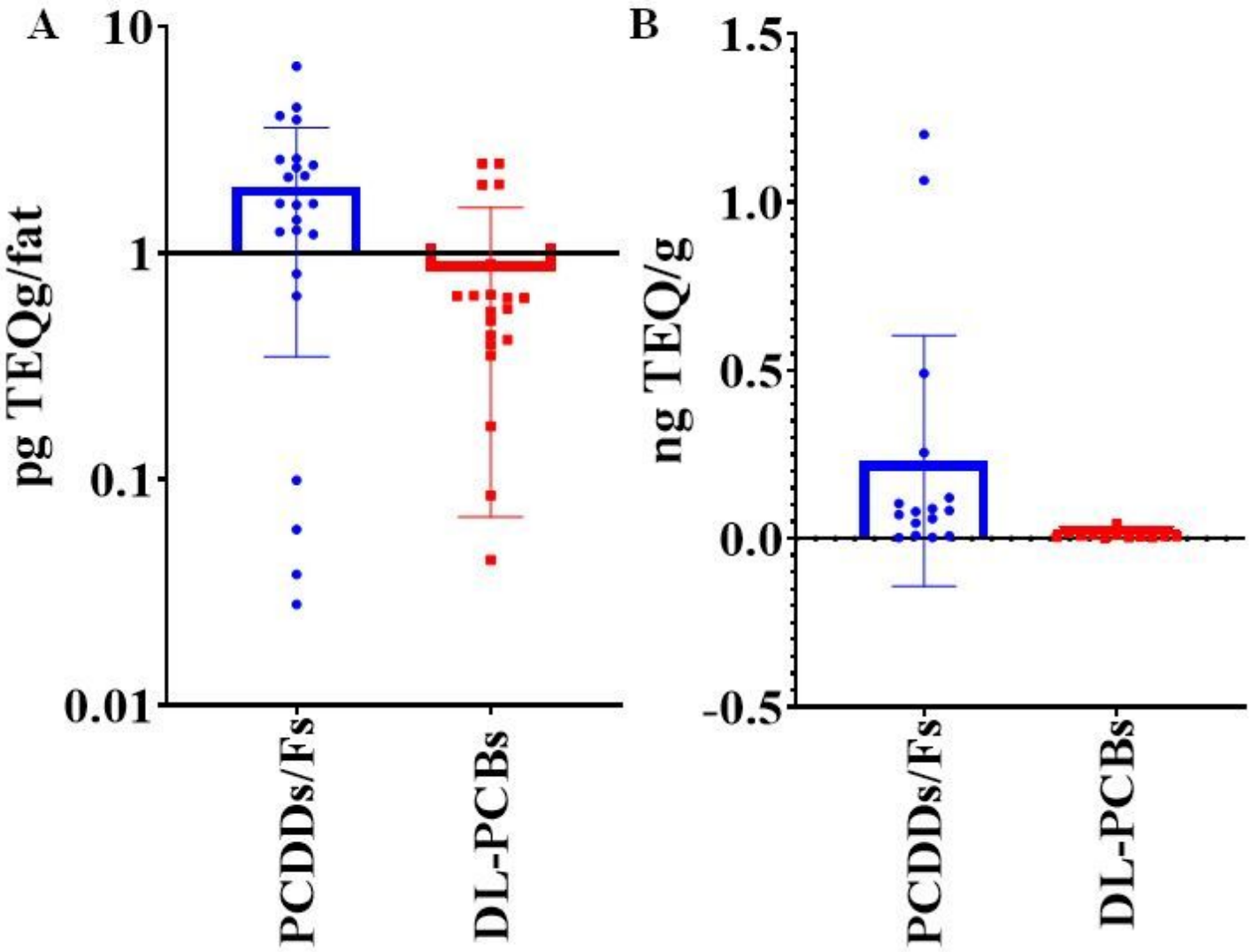

Figure 3

Levels of Total dioxin from bovine milk and Ash samples from different region of Tamil Nadu state, India.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract.jpg