# Spatial trends and human health risks of organochlorinated pesticides from bovine milk; a case study from a developing country, Pakistan

**Saman Sana[(a,b)], Abdul Qadir[a], Mehvish Mumtaz[a,*], Neil P Evans[c], Sajid Rashid Ahmad[a]**

[a] College of Earth and Environmental Sciences, University of the Punjab, Lahore, Pakistan.

[b] Department of Environmental Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan.

[c] Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow, Scotland.

Email: [*]mehvish.env@gmail.com; Phone no: 0092-3324283712

## Abstract

Bovine milk is a nutritious food commodity extensively produced and consumed in Punjab, Pakistan. This study assesses the concentration profile of organochlorine pesticides (OCP; 18 compounds ) in buffaloes and cow's milk in eight major districts of Punjab, Pakistan and the potential impacts of such exposure. The total OCPs in buffaloes and cow's milk samples ranged from 3.93-27.63 ngmL$^{-1}$ and 14.64-77.93 ngmL$^{-1}$ respectively. The overall pattern of mean OCPs concentration in buffaloes and cows milk showed that Hexachlorocyclohexanes (HCHs) are predominant followed by Heptachlors and Dichlorodiphenyltrichloroethane (DDTs). So far, the concentration profile depicted that ∑HCHs, ∑DDTs and ∑Heptachlors did not exceed the maximum residual limits set for buffaloes and cow's milk. The spatial trends in terms of cluster analysis depicted significant variation ($p>0.05$) among the districts in one cluster probably owing to local conditions. Furthermore, recently used DDTs were also identified at some of the selected districts. The risk assessment suggests that the estimated daily intake for each OCP was in accordance with the acceptable daily intake, thus single compound exposure does not pose a significant carcinogenic risk. However, the hazard ratios indicated that the values for ∑DDTs posed risk in adults consuming cow's milk whereas children may face carcinogenic risk on the consumption of both buffalo and cow's milk. The risk may be altered where mixture is considered, furthermore, regarding carcinogenic risks a continuous monitoring based ecological analysis is recommended in the future.

**Graphical abstract**

**Key words**

Cows;

Buffaloes;

Milk;

Organochlorine pesticides;

Pakistan;

Human health risk.

## 1. Introduction

Pakistan is an agrarian country, so the agriculture sector is the backbone of the country's economy (Jaffar et al., 2019). The livestock sub-section of agriculture is growing at high speed in Pakistan and is an integral part of the livelihood and economy for rural community as it is the only sector which provides regular income and readily cashable assets to farming families. In addition to the alleviation of poverty, livestock farming also makes a significant contribution to the Pakistan's foreign exchange earnings and contributes 56.3% to the total agricultural value and 11.76% to the national GDP (Pakistan, 2015). In this regard, Pakistan is the $4^{th}$ largest milk producer in the world, producing 42.17 million tons of milk per annum with buffaloes and cows contributing 62% and 34% of milk production, respectively (Perisic et al., 2015; Ishaq et al., 2018).

Punjab has a leading role in milk production and contributes 25.62 million liters per year to Pakistan's total milk production (Umm e Zia, 2011). Milk is considered a complete natural food and is deemed as an essential food commodity for healthy living of different age groups, especially children, as it contains proteins, fats, lactose, zinc and vitamin $B_{12}$ (Zheng et al., 2014). Milk is also the main constituent of the daily diet, of many vulnerable groups such as infants, school going children and the elderly (Giuseppa Di Bella et al., 2014). According to Food and Agriculture Organization (FAO), milk provides an average 134 kcal of energy capita$^{-1}$ day$^{-1}$ and contributes 8 and 7.3 grams of protein and fat capita$^{-1}$ day$^{-1}$, respectively. However, nutritional components can differ remarkably with geographical production areas (Muhammad Salman et al., 2015).

While it is recognized that pesticides can have an impact on the wider environment (Deti et al., 2014) they are an essential component of modern agricultural technology and have contributed greatly to increased agriculture yields and control of vector-borne diseases. Due to health hazards associated with their use, organochlorine pesticides (OCPs) have been banned or restricted in many countries, but they remain in common agricultural use in many developing countries (Gebremichael et al., 2013; Sajid et al., 2016) due to their low cost, versatility and long half-life (Bedi et al., 2015; Hassan et al., 2015). In 2006, The World Health Organization (WHO) also recommended controlled indoor spray of Dichlorodiphenyltrichloroethane (DDT) in developing countries to combat malaria (Rehwagen, 2006; WHO, 2011). Moreover, the illegal use of OCPs is still reported in many developing countries probably due to their affordability, efficiency and weak regulations (Eqani et al., 2011; Sohail et al., 2018).

To impose bans and apply restrictions on production and usage of OCPs and other hazardous chemicals Stockholm Convention came into being that was globally administrated. Although Pakistan is a signatory of the Stockholm Convention since 2001, OCPs production and use is banned still they are reported in various matrices (Eqani et al., 2011; Chakraborty et al., 2016; Ullah et al., 2019b). In 1950s more than 250 metric tons (MT) of pesticides were imported to Pakistan (Khan et al., 2010). Besides the vast applications, maximum portion of these obsolete pesticides was stored in various areas of Pakistan. According to (Ahad et al., 2010) Pakistan is a country with one of the world's largest stocks of banned/outdated OCPs . A report from FAO states that a program funded by Netherlands is being carried out by the German Agency for Technical Cooperation (GTZ) in Pakistan and 917 tonnes of obsolete pesticides in 133 stores in Punjab has been identified (FAO, 2001). The improper storage practices results in the leakage of these chemicals into the environment (Eqani et al., 2011). The document of Pakistan's National Implementation Plan (NIP) for the Stockholm Convention highlighted the storage of an estimated 6030 million tonnes of obsolete OCPs in the country (Environment, 2004).

The primary route of human exposure to organochlorines is through food and previous studies have demonstrated that animal-origin food is responsible for more than 90% of the average human intake of OCPs (Kannan et al., 1997; Fadaei et al., 2015; Ishaq and Nawaz, 2018). Milk-producing animals can accumulate OCPs from feed sprayed with OCPs either during growth or after harvest (Mumtaz et al., 2015) and/or by inhalation (Deti et al., 2014). OCP concentrations in milk could be further influenced where insecticides are used for the control of insects/pests at dairy farms or milk sale points (Ishaq and Nawaz, 2018). The amount of OCPs that appear in milk, will be influenced by a variety of factors including the nature of the compound, its chemical and physical properties, and the time period for ingestion/exposure (Aslam et al., 2013). The persistence of OCPs in the environment, ability to bio accumulate, biomagnify and capacity to travel from one environmental matrice to another (e.g. from air to soil and water) can also be a reason of milk contamination (Yang et al., 2005). After ingestion, lipophilic OCPs get assimilated from the digestive system into the general circulation. Consequently, diet paves its way for chronic human exposure to some toxic chemicals such as OCPs which can adversely affect health (Tariq et al., 2007; Hassan et al., 2015; Sajid et al., 2016; Yasmeen et al., 2017; Ishaq and Nawaz, 2018; Muhammad Arif et al., 2020). In Pakistan, acceptable limits for OCPs in food have not been documented so Acceptable Daily Intake (ADI) values for OCPs in milk given by USEPA and EFSA is being followed (USEPA, 2007; EFSA, 2013). As milk is the prevalent source of nutrition in Pakistan and OCPs are ubiquitous in the environment so the residues of OCPs can be found in milk which can lead to health hazards. (Riaz et al., 2018; Ali et al., 2020). This study comprehensively investigated OCPs contamination in bovine milk across the major districts of the Punjab. This investigation provides important information about potential human exposure to such contaminants, through the ingestion of dairy products and provides baseline information which may be used to inform policies regarding pesticides use in the agriculture sector.

## 2. Methodology

### 2.1. Material

Analytical grade native standards for OCPs, PCB209 and Tetrachlorometaxylene (TCmX), were purchased from AccuStandard (America). All solutions were stored at -20ºC. Hexane, Dichloromethane (DCM), Ethanol, Acetone were also analytical grade reagents (Merck). Pure Nitrogen gas was purchased from a local gas station (Lahore, Punjab, Pakistan). Solid Phase Extraction (SPE) columns were obtained from SILICYCLE$_{Inc}$. (SPEC-R31830B-06P, CERTIFIED SiliaPrep$^{M}$ C18, 500mg/6mL) and were used for the cleanup. Apparatus used for this study included: micropipette, pipette, sonicator (Model PS-20A), centrifuge machine (Model 800 Electronic Centrifuge), vials stand, rotary evaporator. Glassware used during this study included: centrifugation vials, septa vials and glass tubes.

## 2.2. Sampling strategy

Milk samples from Buffaloes (n=27) and cows (n=30) were collected from eight major districts (Sahiwal, Okara, Kasur, Lahore, Gujranwala, Sialkot, Gujrat, Faisalabad, and Multan) of Punjab, Pakistan. The detailed sampling locations and collected samples are provided in Table S1. To obtain representative samples, sampling was done randomly and milk samples were collected between March to December 2018. Samples were collected in amber glass bottles in an animal's native conditions, and were stored in icebox. The samples were then transported to the Environmental Toxicology Laboratory, College of Earth and Environmental Sciences, University of the Punjab, Lahore and stored at -20 °C until further analysis (Deti et al., 2014; Sajid et al., 2016; Ibigbami et al., 2019). Details of the native conditions, living environment and socio-demographic conditions of the cows and buffaloes were collected via a self-administrated questionnaire (Table S2).

## 2.3. Extraction and Clean up

Extraction of the milk samples was conducted using slight modification of the method described by (Dewan et al., 2013). Milk samples were brought to room temperature and 1 mL of each sample was spiked with 50 µl of TCMX (100 ppb). n-hexane (6 mL) and acetone (3 mL) were added to each sample and were kept overnight in the refrigerator at 4°C. The very next day, OCPs were extracted using a sonicator (Model PS-20A) for 60 minutes at 3°C, followed by centrifugation (3500 rpm; 10 minutes; Model 800 Electronic Centrifuge). The supernatant was separated into another glass vial and the remaining sample extracted twice by similar solvent mixture.

The milk sample may contain several polar impurities (Singh, 2006; Huang et al., 2020), thus the cleanup of extracted samples was conducted with previous method with minor modifications (Kampire, 2011). The extracts were cleaned by SPE using prepared C18 silica cartridges. The cartridges were activated with n-hexane (5 mL), loaded and the extract eluted twice with 5 mL of DCM. The cleaned extract was concentrated through gentle high purity nitrogen streaming (Sosan, 2017), $^{13}$C-PCB 209 (50 µL volume and 100 $ngmL^{-1}$ strength,) was added to a final volume of 1 mL. Samples were filtered (0.22 µm) and stored in the 1.5mL glass vials until the final analysis.

### 2.4. Instrumental Analysis

Analysis of all samples was performed on an Agilent Gas Chromatography Mass Spectrometer (Agilent Technologies 5975C inert XL EI/CI MSD with Triple Axis detector; 7890A GC System) accompanied with Auto Sampler (Agilent Technologies 7693) at the Environmental Biotechnology Laboratory of the University of Glasgow, United Kingdom. Selected Ion Monitoring (SIM) mode was used for the analysis of 18 different OCPs (Aldrin, α-HCH, β-HCH, γ-HCH, δ-HCH, trans-Chlordane, cis-Chlordane, 4,4'-DDD, 4,4'-DDE, 4,4'-DDT, α-Endosulfan, β-Endosulphan, Endosulfan Sulfate, Heptachlor, Heptachlor Epoxide (Isomer B), Methoxychlor, cis-Nonachlor and trans-Nonachlor). The sample injection volume was 1 µL. Varian column (CP-Sil 8CB, 50 m, 0.25 mm, and 0.25 µm.) was used for sample analysis. During the analysis, the injector port temperature was kept at 250 °C. The source temperature of MSD (mass spectrometric detector) was 230 °C and 150 °C was the quadruple temperature. The following scheme was adopted for the analysis of each sample: for the first 3 min the temperature was 150 °C, 4 °C/min to 290 °C, and the isothermal process was kept for 10min.

### 2.5. Quality control and quality assurance (QC/QA)

To ensure the accurate quantification of OCPs, all procedures for quality control were observed strictly. Glassware was dried at 450°C for approximately 6 h after washing and rinsing with distilled water and DCM respectively. Standards of 1 ng $mL^{-1}$ 10 ng $mL^{-1}$, 20 ng $mL^{-1}$, 50 ng $mL^{-1}$, 100 ng $mL^{-1}$, 200 ng $mL^{-1}$, 500 ng $mL^{-1}$ and 1000 ng $mL^{-1}$ were used for the calibration curves and instrument calibration. The limit of detection and quantification were calculated (Table S7). The corresponding dectection limit was 3 times of the signal to noise ratio (S/N), while the quantification limits were ten times of the S/N. The samples were analysed in small sets and after each set of 10 samples, a procedural blank was run. The field, procedural and solvent blanks were all below detection limits. The surrogate recovery for TCmX ranged between 75 and 84% for all samples. The calculated spiked recoveries were 83-130% (mean= 112%). The calculated relative standard deviation of the spiked replicates was less than 20% (mean= 8.04 %). The quality of the data was assured and the Agilent MSD productivity Chemstation software was used for peak integration and data analysis.

### 2.6. Human Health Risk Assessment

For the calculation of carcinogenic and non-carcinogenic human health risk USEPA guidelines were followed (Dougherty et al., 2000; Sosan, 2017). The risk was calculated for both children and adults. For non-carcinogenic health risks, estimated daily intake (EDI) of milk was compared with the ADI. For the carcinogenic human health risks, the hazard ratio (HR) was calculated. Average body weight was used for the calculations of EDI and the human risk level along with the concentration of OCPs and daily milk consumption (Dougherty et al., 2000).

#### 2.6.1. Non-carcinogenic risk assessment

The following formula (equation 1) was used to calculate EDI.

$$EDI = \frac{CR \times C}{BW} \qquad (1)$$

Where,

CR is the consumption rate (mL $d^{-1}$) (Pakistan Economic Survey, 2018)

C is the quantified concentration (ng $mL^{-1}$) of selected OCPs isomers

BW (kg) is average bodyweight (children = 27.7 kg, adults = 60 kg) (Sosan, 2017; Adeleye, 2019)

### 2.6.2. Carcinogenic risk assessment

The formula used to calculate HR was given by (Dougherty et al., 2000) as shown in equation 2. Oral Slope Factor (OSF) values were obtained from the Integrated Risk Information System (IRIS), United States Environmental Protection Agency (USEPA) for each contaminant (USEPA, 2007).

$$HR = EDI/CBC \quad (2)$$

Where,

EDI (ng $L^{-1}$ $d^{-1}$) is estimated daily intake

CBC (ng $L^{-1}$ $d^{-1}$) is the cancer bench mark concentration.

CBC was calculated by

$$CBC = (RL \times OSF \times BW)/CR \quad (3)$$

Where,

RL is the risk level ($10^{-6}$)

OSF (mg $kg^{-1}$ $d^{-1}$ ) is the oral slope factor

BW is average body weight (children = 27.7 kg, adults = 60 kg)

CR is the consumption rate (mL $d^{-1}$)

## 2.7. Statistical analysis

Descriptive statistics were applied to all milk samples collected from different districts. Samples were further analyzed by Cluster analysis and Krushkal Wallis Test to check differences in the OCPs concentration between different districts. P value > 0.05 was considered to be statistically significant. Microsoft Excel version 2010, Statistica and IBM SPSS were used to calculate the detailed descriptive (mean, $50^{th}$, $90^{th}$ percentiles and range). The graphs were prepared by using Origin (Pro 8). Furthermore, Arc GIS (version 10.2) was used to show the spatial distribution of OCPs concentration profile in the different districts of Punjab, Pakistan.

# 3. Results and Discussion

## 3.1. OCPs profiles in the bovine milk samples

At least one OCP isomer was detectable in each of the analysed samples (n=57) Fig 1a. For Buffaloes, the Hexachlorocyclohexanes isomers (∑HCHs) were predominant (detection frequency of α-HCH =100%, β-HCH = 93%, γ-HCH = 100%, δ-HCH = 78%), followed by ∑DDTs (detection frequency of 4-4'DDE = 48%, 4-4'DDD = 37%, 4-4'DDT = 30%) and ∑Heptachlors isomers (detection frequency of Heptachlor = 85% and Heptachlor Epoxide = 22%). For cows, the ∑HCHs isomers were predominant (detection frequencies of α-HCH = 100%, β-HCH = 90 %, γ-HCH = 100%, δ-HCH = 87%), followed by isomers of ∑DDTs (detection frequencies of 4-4'DDE = 27%, 4-4'DDD = 43%, 4-4'DDT = 20 %) and ∑Heptachlors isomers (detection frequencies Heptachlor = 87 and Heptachlor Epoxide = 30). The predominance of HCHs is in agreement with the study reported from Spain where HCHs were present in 100% of the milk samples analyzed (Luzardo et al., 2012).

### 3.1.1. Concentration profile of OCPs in the Buffaloes milk

The detailed descriptive statistics of 18 selected OCPs in the buffaloes milk samples are given in Table S3. The mean concentration of ∑OCPs was 15.76 ng $mL^{-1}$ with a range of 3.93 – 27.63 ng $mL^{-1}$. Among the selected OCPs, ∑HCHs showed the highest rate of contamination with a 67% percent contribution to total OCP load and a mean concentration of α-HCH = 1.32 ng $mL^{-1}$, β-HCH = 1.39 ng $mL^{-1}$, γ-HCH = 7.03 ng $mL^{-1}$, δ-HCH = 0.75 ng $mL^{-1}$. The isomers profile of the present study depicted that, among ∑HCHs, the residual level of γ-HCH was the highest (range: 0.69-19.44 ng $mL^{-1}$, percent contribution: 44.59 %). These HCH isomer concentrations are lower than those reported in buffalo in Turkey i.e. α-HCH: 6.34 ng $mL^{-1}$, β-HCH: 20.41 ng $mL^{-1}$, γ-HCH: 9.77 ng $mL^{-1}$, 4-4'DDE: 4.36 ng $mL^{-1}$ and 4-4' DDT: 13.54 ng $mL^{-1}$ (Atmaca, 2019). The detailed comparisons are provided in Table S4. ∑Heptachlors and ∑DDTs were the second and third most abundant isomers among all the OCPs with a percent contribution of 9.70 % and 9.51 % respectively. The mean concentration of Heptachlor was 1.44 ng $mL^{-1}$ and ranged between 0.14-6.32 ng $mL^{-1}$ with Heptachlor being the predominant (23%) isomer followed by Heptachlor epoxide (6%). These results contrast with those of a study conducted in Brazil on bovine pasteurized milk which found the highest mean concentration levels in ∑DDTs closely followed by ∑Heptachlors i.e. 3.74 ng $mL^{-1}$ and 3.05 ng $mL^{-1}$ respectively (Avancini et al., 2013). This high concentration of Heptachlors in samples collected from Punjab in the current study could be due to its continued use as insecticides in this area, but its presence can also be related to surface runoff from urban areas, municipal and industrial effluents, and atmospheric deposition (Zhou et al., 2006; Eqani et al., 2011).

The mean concentration of DDTs was 1.50 ng $mL^{-1}$ with 9.5% (percent contribution ) among all the OCPs. The isomers profile indicated that 4,4'-DDD had the highest average concentration with a value of 0.97 ng $mL^{-1}$ followed by 4,4'-DDT and 4,4'-DDE with mean values of 0.41 ng $mL^{-1}$ and 0.12 ng $mL^{-1}$ respectively.

∑Chlordanes showed 3.84% contribution among all the analyzed OCPs. Its isomers trans-Chlordane and cis-Chlordane showed mean concentrations of 0.39 and 0.21 ng $mL^{-1}$. Previously a study conducted in 2015 in India showed mean concentrations in buffalo milk of trans-Chlordane and cis-Chlordane of 0.66 ng $mL^{-1}$ and 0.17 ng $mL^{-1}$ respectively. The percent contribution of ∑Endosulfans among all the OCPs in the current buffaloes milk samples was 1.26%, with isomeric concentrations of 0.14, 0.06 ng $mL^{-1}$ and not detected (ND) for Endosulfan Sulfate, Endosulfan-I and Endosulfan-II, respectively. When compared with a previous study conducted in Brazil in 2013 on bovine milk samples, values of Endosulfans were found to be higher than the present study with mean values 1.95 ng $mL^{-1}$ and 2.23 ng $mL^{-1}$ for Endosulfan-I and Endosulfan-II respectively (Avancini et al., 2013). Yet another study conducted in India in 2008 showed a higher mean value of ∑Endosulfans as 49.2 ng $mL^{-1}$, its isomers followed the following trend Endosulfan-II; 23 ng $mL^{-1}$ > Endosulfan sulfate; 19.8 ng $mL^{-1}$ > Endosulfan-I; 6.5 ng $mL^{-1}$ (Nag and Raikwar, 2008). ∑Nonachlors showed a percent contribution of 1% among all the selected OCPs. Its isomers trans-Nonachlor and cis-Nonachlor showed mean concentration values as 0.16 ng $mL^{-1}$ and ND respectively. This value is lower than another study conducted in Brazil in 2013 where trans-Nonachlor showed mean concentration 2.05 ng $mL^{-1}$ (Avancini et al., 2013). Methoxychlor contributed 1.20% among all the OCPs with a mean value 0.19 ng$mL^{-1}$ and range (0.00-0.47 ng$mL^{-1}$). The reported concentration was higher than a previous study conducted in Brazil in 2013 where Methoxychlor showed comparable concentration (Avancini et al., 2013). Aldrin showed a relatively higher percent contribution (6.91%) among all the selected OCPs. The mean concentration of Aldrin is 1.09 ng $mL^{-1}$ and range 0.52-2.25 ng $mL^{-1}$ in buffaloes milk. The mean value of Aldrin was slightly higher thanthe mean value (0.99 ng $mL^{-1}$) reported in Turkish buffaloes milk samples (Atmaca, 2019). On the whole, OCPs in buffalo milk samples in Punjab, Pakistan have either higher or lower mean concentrations as compared to the OCPs level in milk of other countries(Table S4). One of the possible cause for this trend maybe human activities like use of pesticides or leakage from the old stocks of obsolete pesticides (Ali et al., 2020). While the low concentration of some OCPs detected in milk samples of buffaloes is probably because of the prevailing local conditions of Punjab, Pakistan (Neelam Shahzadi, 2013).

**3.1.2. Concentration profile of OCPs in the cow's milk**

The mean concentration of OCPs in cows milk was 44.82 ng $mL^{-1}$ with a range of 14.64-77.93 ng $mL^{-1}$ (Fig 1b). The homologue profile showed that among the selected OCPs, HCHs were predominant (74%) followed by ∑DDTs (11%), ∑Heptachlors (7%), ∑Endosulfans (3%), Aldrin (2.56%), ∑Nonachlors (2.20%), ∑Chlordanes (0.73%) and Methoxychlor (0.40%).

The HCHs isomer profile indicated that γ-HCH or Lindane showed highest mean concentration (22.43 ng $mL^{-1}$) followed by δ-HCH (3.87 ng $mL^{-1}$) and β-HCH (3.5 ng $mL^{-1}$). Lindane is considered as the most active and stable isomer of HCHs (Raslan, 2018). The mean ∑HCH concentrations observed in this study differed from the profile reported from India, where only one isomer γ-HCH was detectable but the concentration of γ-HCH was significantly greater with a mean value 900 ng$mL^{-1}$ (Bedi et al., 2015). Whereas the reported γ-HCH concentrations in Iran (13.49 ng $mL^{-1}$) was comparable (Bayat et al., 2011) to the current study and were only slightly lower than those reported in Nigeria; HCHs range from 4-144 ng $mL^{-1}$ (Ibigbami et al., 2019).

Among metabolites of ∑DDTs (mean concentration: 4.76 ng $mL^{-1}$), the observed mean concentration trend was 4,4'-DDD (2.64 ng $mL^{-1}$) > 4,4'-DDT (1.88 ng $mL^{-1}$) > 4,4,-DDE (0.23 ng $mL^{-1}$). The concentrations of DDTs (4.76 ng $mL^{-1}$) in the present study were lower than those reported for some DDT isomers in samples of cows milk from India i.e. 2,4'-DDE 0.093 ng $mL^{-1}$; 2,4'-DDD 0.775 ng $mL^{-1}$ (Bhuvaneshwari et al., 2015), and two studies from Iran in 2009 and 2011 where the observed values were 4,4'-DDT; 51 ng $mL^{-1}$ >4,4'-DDD; 28 ng $mL^{-1}$ > 4,4'-DDE; 17 ng $mL^{-1}$, 4,4'-DDE; 7.44 ng $mL^{-1}$ > 4,4'-DDT; 6.83 ng $mL^{-1}$ > 4,4'-DDD; 2.81 ng $mL^{-1}$ respectively (Ashnagar, 2009; Bayat et al., 2011). The detailed comparisons are shown in Table S4. In contrast to these studies, a study conducted in India in 2015 reported concentrations of DDT in fresh milk samples with a mean value 1.6 ng $mL^{-1}$ (Bedi et al., 2015) which is almost in agreement with the findings of the present study.

Cis-Nonachlor and Endosulfan-II were not detected in any of the analyzed milk samples. The inability to detect ∑Endosulfans in milk samples might be restriction on its use in Pakistan (Hassan et al., 2015). Aldrin was detected in samples with a mean concentration of 1.15 ng $mL^{-1}$. This concentration is higher than the studies conducted in Romania and Egypt; 0.63 ng $mL^{-1}$ and 0.382 ng $mL^{-1}$ respectively (Farag Malhat, 2012; M. Miclean et al., 2017) but is much lower than a study conducted in Nigeria in 2019; 29 ng $mL^{-1}$ (Ibigbami et al., 2019).

The presence of high percentages of various OCPs in the bovine milk suggests that animals are still being exposed to OCPs, the most likely route being through contaminated feedstuffs, either as a result of the fact that many of these pesticides remain in the market even after being banned or due to their storage and use or their persistence in the environment after use (Rainbow Vogt et al., 2012). Among all of the selected OCPs, most of the compounds were in accordance with MRLs. However, the observed concentration of OCPs could be due to their extensive use, particularly during the cultivation of seasonal crops(Khan et al., 2020). High concentrations of OCPs in the bovine milk revealed the increase and improper use of these pesticides by the farmer. A continuous monitoring program is needed in future to check the profile of these compounds in animals milk and other environmental components.

**3.2. Spatial distribution patterns of OCPs in the bovine milk**

Statistical analysis indicated the concentration of OCPs differs significantly ($p>0.05$) between various districts of Punjab, Pakistan. Cluster analysis (Figure 2a) indicted two clusters in terms of buffaloes milk, group 1 includes the districts; Sahiwal, Okara, Kasur, Lahore and Sialkot, whereas group 2 includes Gujrat, Faisalabad and Multan. Furthermore, three groups of cities were identified with regard to OCPs in cows milk (Figure 2b). Cluster 1 represented Sahiwal and Okara, cluster 2 Kasur, Lahore, Gujranwala and the third cluster included Sialkot, Faisalabad and Multan. The reason for the different clusters might be the use of a similar type of pesticides on crops, analogous topographic features and environmental behavior of study sites (Osman Tiryaki, 2010). Furthermore, the soils that were heavily treated with OCPs in the past are still a good source of OCPs (Fiedler et al., 2000). Previously, several researchers have also reported OCPs in soils of Pakistan. (Syed and Malik, 2010; Jabir et al., 2013; Syed et al., 2013; Ali et al., 2020).

The spatial distribution trends (Figures 3a and 3b) depicted that the Okara district showed the highest level of OCPs (27.625 ng $mL^{-1}$) in buffaloes milk followed by Kasur and Multan with a mean concentration of 25.4 ng $mL^{-1}$ and 24.19 ng $mL^{-1}$ respectively. Concerning cows milk, Kasur district showed the maximum mean concentration (77.93 ng $mL^{-1}$). Multan and Gujranwala showed nearly the same contamination status with a mean concentration of 60.99 ng $mL^{-1}$ and 59.82 ng $mL^{-1}$ respectively. While the cows milk from Okara district also contains higher values of the OCPs (54.88 ng $mL^{-1}$). Okara district has both fertile agricultural lands and a number of agriculture-based industrial units. Large quantities and variety of pesticides and insecticides usage had been reported in this area (Rafique et al., 2016). The district is also one of the best milk producing districts of Punjab (Muhammad Naeem Tahir, 2019) thus supports the findings of the present study.

### 3.3. Relevant sources of OCPs in the bovine milk

In this study, samples from different local environment such as urban areas and dairy farms, showed the presence of OCP residues in milk. Based on the isomer specific ratio, it has been reported previously that it is possible to trace the sources of ∑HCHs by using the ratio α-HCH/ γ-HCH. If the ratioα-HCH/ γ-HCH is greater than 4 it will 4 suggest the fresh input of HCH and vice versa (Jabir et al., 2013). The α-HCH/γ-HCH value less than 3 indicates Lindane as a source of HCHs, while the α-HCH/γ-HCH ratio ranging between 3 and 7 is an indicator of technical HCHs (Sun et al., 2010).

In the present study the ratio of α-HCH/ γ-HCH for all the samples were less than 4 which suggested the historic use of HCH mixture. Moreover, the percentage of γ-HCH > α-HCH, ranging between 0.09 - 1.07 indicates usage of Lindane or γ-HCH.. Lindane is widely used in Pakistan for agricultural applications ( Baqar et al., 2018), also for medicinal purpose as scabicide (Daud et al., 2010; Ishaq and Nawaz, 2018), and possibly the reason for the high detected HCHs in the milk of buffaloes and cows of selected districts.

The ratio 4,4'-DDT/(4,4'-DDE + 4,4'-DDD) can also provide a measure of historical and more recent use <1 suggests the inputs of technical DDTs while values >1 suggest recent use (Jabir et al., 2013). The buffaloes milk samples from all the studied districts of the present study except Lahore showed values less than 1 which implies that recent application of DDTs is still being done in Lahore. . For cow milk samples, the calculated ratio was greater than 1 in Kasur, Gujranwala and Multan districts which indicated the recent use of DDTs whereas other districts didn't show results for recent use of DDT.However, the detection frequency of DDTs was less than 50% so it should be carefully interpreted while comparing with previous work. So far, previous studies from Pakistan confirmed the usage of DDTs in different environmental matrices as insecticide and pesticides (Huma Naeem, 2014; Randhawa et al., 2016). DDT can be biodegraded into DDD by dechlorination (a reduction process) under anaerobic conditions and to DDE under aerobic conditions through dehydrochlorination, an oxidative process (Jabir et al., 2013). Since DDT is known to undergo metabolic conversion and dehydrochlorination, the presence of metabolites of DDT *i.e.* DDD and DDE encountered in a previous study that is in agreement with the current work might be due to reported processes (Aslam et al., 2013). The DDD/DDE ratio (Eqani et al., 2011) also exceeded one for some districts for both buffaloes and cows, highlighting the high proportion of DDD in the environment and indicating anaerobic environmental conditions (Ullah et al., 2019a) in district Kasur, Sialkot, Gujrat and Multan. A high DDT/DDE ratio (>1) symbolizes chronic and an ongoing exposure of DDT. Conversely, a low DDT/DDE ratio (<1) implies high environmental persistence and continuing bioaccumulation (Ahlborg et al., 1995; Jaga and Dharmani, 2003). In the present study, a high value of DDT/DDE ratio was observed in some districts including Kasur, Lahore, Gujranwala and Multan.The detailed calculated ratios are presented in Table S5.

### 3.4 Human Health risk

#### 3.4.1. Non-Carcinogenic risk

Non-Carcinogenic risk can be evaluated by comparing EDI with an ADI for OCPs (Dougherty et al., 2000; Wang et al., 2011). The obtained values for EDI of investigated OCPs, through consumption of milk, for adults and children in Punjab province of Pakistan, were far below ADI values set by the European Food Safety Authority (EFSA) and also from the values set by joint interaction of Food and Agricultural Organization (FAO) and World Health Organization (WHO) (FAO and WHO, 2002; EFSA, 2013) for different compounds which are given in Table S6. For each investigated OCP for milk consumption, the EDI varied ($EDI_{children}$> $EDI_{adults}$).

In the present study, Aldrin had value below ADI i.e. 10.90 ng mL$^{-1}$ d$^{-1}$ for adults and 23.61 ng mL$^{-1}$ d$^{-1}$ for children in buffaloes. Similar trend was noticed for the cows milk i.e. 11.50 ng mL$^{-1}$ d$^{-1}$ in adults and 24.91 ng mL$^{-1}$ d$^{-1}$ in children. HCHs, DDD and DDT depicted relatively higher values as compared to other studied OCPs but all the values were lower than the ADIs. The comparison depicted that EDI of the present study was lower than the values given in previously published study from Egypt in Buffaloes and Cows milk (Raslan, 2018).. On the contrary, a study conducted in Romania (M. Miclean et al., 2017) showed lower EDIs for adults i.e male (α-HCH=0.00034, β-HCH=0.00138, γ-HCH= 0.00039, 4,4'-DDE= 0.00142, 4,4'-DDD= 0.00002, 4,4'-DDT= 0.00008, Endosulfan-I= 0.00009 and Endosulfan-II= 0.00007 μg kg$^{-1}$ d$^{-1}$), female (α-HCH=0.0004, β-HCH= 0.00180, γ-HCH= 0.00051, 4,4'-DDE= 0.00184, 4,4'-DDD = 0.00003, 4,4'-DDT= 0.00009, Endosulfan-I= 0.00011, and Endosulfan-II= 0.00009 μg kg$^{-1}$ d$^{-1}$) and children (α-HCH=0.00185, β-HCH=0.00748, γ-HCH= 0.00213, 4,4'-DDE= 0.00768, 4,4'-DDD= 0.00011 and 4,4'-DDT= 0.00008, 0.00009, 0.00039, Endosulfan-I= 0.00009, 0.00011, 0.00046 and Endosulfan-II= 0.00038 μg kg$^{-1}$ d$^{-1}$which is equal to ng mL$^{-1}$ d$^{-1}$) respectively as compared to the present work (Table S6). Studies conducted in Turkey (Bulut et al., 2011) showed an agreement with the current work. Thus, the humans in the major districts of Punjab, Pakistan may be safe, nevertheless the sample size, number of studied OCPs, geographical location and the possibility that OCPs act differently in a mixture,can not be neglected.

#### 3.4.2. Carcinogenic risk

The calculated HR greater than 1 revealed the potential human health risks (Dougherty et al., 2000). Table 1 provides the HR calculated for the current study in adults and kids. It was observed that γ-HCH, DDD and ∑DDTs exhibited HR values > 1 in adults for cow milk samples. In 50th value δ-HCH has value greater than 1 and for 90th percentile of cow milk values for adults, δ-HCH, DDD, DDT and ∑DDTs show carcinogenic risk, whereas, the HR values of DDDfor 90th percentile showed values greater than 1 in case of buffaloes milk. In case of children consuming cow's milk, cancer threat (HR value > 1) was observed. forγ-HCH, δ-HCH, DDD, DDT and overall ∑DDTs... Although, risk calculated for Aldrin in children is less than 1 (Table 1) but owing to its carcinogenicity, neurotoxicity and lowest ADI (0.0001 mg $kg^{-1}$) it cannot be neglected(Lozowicka et al., 2014).

The results of the present study were compared to a previous study on milk samples acquired from Greek markets . In this work DDTs showed highest HR values in children of 1-3 years age (Tsakiris et al., 2015). Another study conducted in Egypt showed no carcinogenic risk in milk samples of buffaloes and cows (Raslan, 2018). Similarly, a study conducted in Nigeria showed no potential health risk to the human population consuming cows' milk (Ibigbami et al., 2019). The observed varying trend might be due to different local and geographical features.

The values of HR higher than one suggest a potential risk to human health, especially health of children. Risk posed to the health by individual congeners and isomers may have different potency as compared to the risk posed by mixture of OCPs. Thus the results for human health risk assessment should be considered and taken seriously by environmental protection agencies and agricultural governmental authorities. It is also recommended to do continuous monitoring based studies in future so that fresh inputs could be traced and these health risks could be avoided.

**Conclusion**

The present study highlights the concentration profile, health risk and sources of OCPs in the Bovine milk from Punjab, Pakistan. The quantified OCPs depicted that, ∑HCH was the dominant OCP with comparatively higher concentration as compared to national and international studies followed by DDTs and Heptachlors. The spatial distribution patterns highlighted that regarding isomers, highest HCHs were reported from Kasur and Multan. While the maximum concentration of ∑DDTs was in Lahore. This significant variation could be due to native conditions. The investigation of source reported the fresh inputs of DDTs in Lahore followed by Kasur and Gujranwala and Multan. Except Lindane all the selected HCHs were used historically. Noncarcinogenic risk for selected OCPs for both buffaloes and cows was within the available acceptable risk levels, whereas γ-HCH/Lindane and ∑DDTs pose a considerable carcinogenic threat to the children consuming milk of either buffaloes or cows. Pakistan is lacking in such continuous monitoring surveys; thus, detailed investigation is recommended in future by governmental sector.

**Acknowledgment and conflict of interest**

Authors highly acknowledge the people who helped during sampling and all the technical/ laboratory staff at the School of Engineering and Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow, Scotland, for their assistance during GC/MS instrumental analysis. Authors also acknowledge the input of Dr. Ambreena Mubashir, (Department of Environmental sciences, Kinnaird college for women university, Lahore, Pakistan) in the finalization of geographical maps.

The authors declare no conflict of interest.

**Supplementary material**

The related additional information can be found in the attached document.

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