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Environmental contamination by Hexachlorocyclohexane of bovine milk: a case study in Central Italy.

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ABSTRACT

Hexachlorocyclohexane (HCH) contamination of agro-ecosystems is a World scale problem. At the beginning of 2005 a wide area in Central Italy was discovered to be polluted by HCH (mean concentration in soils 0.078 mg HCH/kg). Few information are available on HCH metabolism in dairy cows. For this reason, a study was carried out to assess the presence of HCH in bovine milk after an event of chronic exposure to HCH. During the experimental phase, animals were fed forages and concentrates containing no detectable HCH. In five dairy farms, milk and blood samples were collected from July to September 2005 every three weeks from animals in different lactating stage. Pre-treated samples were analysed by gas-chromatography using an Electron Capture Detector for quantification of HCH-isomers. Data were statistically analysed by ANOVA and LR. Differences in milk contamination by β-HCH among dairy farms ($P < 0.01$) and sampling time were observed ($P < 0.05$). Milk contamination levels exceeded occasionally the EU limit (0.003 mg β-HCH/kg), posing potential hazards for human consumption. A linear regression between blood serum and milk β-HCH content was found ($r^2 = 0.919$, $P < 0.05$). Furthermore, β-HCH as a traces was detected in blood serum of animals when milk levels were below the analytical limits indicating the possible usefulness of blood HCH determination as an early indicator of animal exposure to that contaminant.

INTRODUCTION

Hexachlorocyclohexane (HCH, also called benzene hexachloride - BHC) is an organochlorine pesticide (OCP) used world-wide since the late 1940s (Li *et al.*, 1998) and is available in two formulations: technical HCH as a mixture of different isomers (α-, β, γ, δ and ε-HCH) and Lindane (more than 90% as γ-HCH) (UNEP, 1983). Although only γ-HCH shows insecticidal properties, all isomers are acutely and chronically toxic for animals. For α-HCH and β-HCH, two no insecticide isomers, it was reported to have reproductive and endocrine-disrupting effects (Van Velsen *et al.*, 1986). The utilization of HCH and other OCP contributed to increase agricultural yield, protect livestock, and to control vector-transmitted diseases (Li *et al.*, 1996). However, over the past decades due to an indiscriminate usage of these compounds, a global contamination has been found to be ubiquitous and persistent in several environmental media and biota (Woodwell *et al.*, 1971; Chowdhury e*t al.*, 1987; Tanabe, 1988; Tanabe *et al*., 1999). In European Countries ban or severe restriction of HCH usage are in force since the last years of 1970s (Breivik *et al.*, 1999). Nevertheless, previously

production of Lindane for commercial purposes leaded to a high quantity of process discharges. Due to these facts, large amounts of HCH without insecticide effects (α-, β-, δ- and ε-isomers) discarded during the purification process of Lindane, have been disposed for years into dumps around industrial sites or, even worse, just buried in rural areas. Disposal practices may imply great risks for environmental contamination, and for humans health by food chain transfers and pesticides bio-accumulation. Indeed, the persistence and fat solubility make this class of compounds one of the more serious cause of residue problems in animals and animal products (Fries, 1977). At the beginning of 2005, the Sacco river valley, a wide area of dairy cows farming in the province of Rome (Latium, Central Italy), was interested by an episode of environmental contamination by HCH. An official survey of the Sacco river waters and sediments, showed a wide pollution by HCH due to a suspected industrial landfill leakage. As a consequence, soils, forages and milk from several dairy farms near the path of Sacco river, were found contaminated by β-HCH sometimes exceeding the Italian and European law limits. Actually, to our knowledge, no studies has been done to improve the understanding on HCH metabolism in dairy cows under field conditions. Therefore, the aim of the present study was to assess the field scale factors influencing the presence and the excretory kinetics of β-HCH in milk of cattle after an event of accidental chronic exposure via the food chain. As stated by Otero *et al.* (1997) blood is an ideal medium of body burden estimation for β-HCH isomer, the dominant one in this tissue (Murphy & Harvey, 1985; To-Figueras *et al.*, 1997; Waliszewski *et al.*, 2004), because it gives information on this organochlorinated compound accumulated in fat and it is relatively easy to obtain and analyze. Taking in account these facts, the research also aimed to ascertain in cattle the association between milk contamination and blood serum presence and level of the β-HCH isomer, as a sensitive marker of cattle exposure to HCH environmental contamination and as an early indicator of milk production potentially hazardous for human consumption.

MATERIAL AND METHODS

Sampling

Five farms were selected (Fig. 1) in the polluted floodplain of the Sacco river, on the basis of a previously official survey (IZSLT, 2005) of milk and soil contamination by β-HCH (mean concentration in soils of 0.078 mg HCH/kg). In these farms, milk and blood samples were collected from July 2005 to September 2005 every three weeks from animals at different lactation phase (1st: from 10 to 100, 2nd: from 101 to 200, and 3rd lactation: last 100 days in milk) and parity (from first to sixth lactation). From 3 to 9 animals were selected for β-HCH screening in different farms. Individual milk samples were taken at morning milking using calibrated weigh jars. Milk samples were stored at -20°C until analysis. Venous blood samples were obtained using vacutainer tubes without anticoagulant agent (Becton, Dickinson & Company, Plymouth, UK). The samples were left naturally coagulate at 4°C and then centrifuged to separate the serum which was stored at -20 until analysis. During the study, animals were administrated feedstuffs containing no detectable HCH.

Laboratory analyses

Milk and blood samples treatment and clean-up

Milk samples (20 ml) were extracted with 40 ml acetone-petroleum ether mixture (Sigma, Germany) by shaking. After a preliminary volume reduction of the extract a clean-up step was

performed using Florisil® as adsorbent matrix and the purified extract concentred to 2 ml under a pure nitrogen stream (Rivoira, Italy) for submission to GC-ECD analysis.

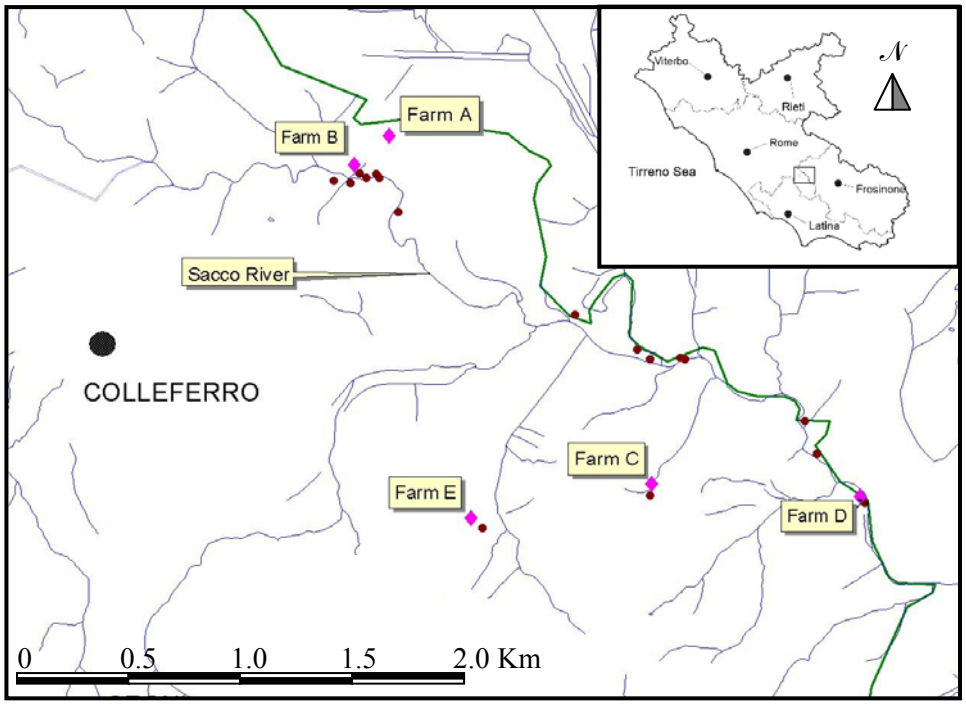

Figure 1 – Position of the dairy farms investigated in the Province of Rome (green solid line). Brown dots indicate farm soils cultivated for forage production.

Blood serum treatment were performed as reported by Otero *et al.* (1999). Briefly, 2 ml of blood serum were digested adding 3 ml of n-hexane (Sigma-Aldrich, Germany) and 2 ml of concentrated sulphuric acid (Sigma-Aldrich, Germany) and then stirred for 30 s. After the mixture was cooled to room temperature, five drops of acetone were added to help phase separation. The supernatant n-hexane were poured out and collected in a polyethylene 10 ml tube. The remaining sulphuric solution was re-extracted two more times with 2 ml of n-hexane using always acetone as phase separation enhancer. The 7 ml n-hexane phase collected was washed with 2 ml of sulphuric acid and then concentrated to almost dryness under a gentle pure nitrogen stream (Rivoira, Italy) at room temperature. Prior to be submitted to GC-ECD analysis, cleaned-up samples were recovered with iso-octane (Merk, Darmstad Germany) into 1,0 ml micro-tubes to a 500 µl final volume.

HCH determination in milk and blood samples

Pre-treated milk and blood samples were analysed using a Hewlett-Packard 5890A model gas-chromatograph provided with an ECD system for detection of HCH isomers and a 30 m x 0.53 mm I.D., 1.5 µm film thickness DB1 capillary column (J&W Scientific, Folsom, CA, USA). A second 30 m x 0.53 mm 1.0 µm film thickness DB17 capillary column (J&W Scientific, Folsom, CA, USA), was used as a confirmative column. The GC oven temperature was programmed to heats columns at 140°C (holding time 2 min) and then rises to 180°C at 6°C/min, 195°C at 0.8°C/min and 280°C at 30°C/min, keeping the last temperature for 10 min'. The injector and detector temperature were respectively 280°C and 300 °C. Injection (1.5 µl) was operated in split/splitless mode and Helium (Rivoira, Italy) was used as carrier gas (20 ml/min) and injector make up gas (10 ml/min). Mean recovery for β-HCH from milk

was 90% while for blood serum was 72%. Detection limits (LOD) (DIN, 1994) were respectively 0.0002 mgβ-HCH/kg for milk and 0,0002 μgβ-HCH/ml in the case of blood serum. Quantification limits (LOQ) were set as 3.1 times the respective LODs.

Climatological data

During the study, daily temperature (minimum and maximum) and rainfall data are obtained from the A.R.S.I.A.L. Meteorology Station situated at Anagni (Province of Frosinone, Latium, 352 m of altitude), less than 7 km away from the study area. Hereafter, data are expressed as mean (temperature) and cumulative (rain) values per decade.

Statistical analysis

Data were analyzed by Nested ANOVA to explore causal factors driving milk contamination. Linear Regression was performed to study milk vs. blood contamination by β-HCH. Effects were taken in account as significant at the most for P-level under 5% ($P < 0.05$). To verify the normality of data distribution, a preliminary evaluation was performed applying a Shapiro & Wilks Test (Shapiro & Francia, 1975). Data analysis was performed using the statistical software package STATISTICA™ Release 6.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Milk contamination by β-HCH

The contamination of milk by β-HCH, grouped for sampling dates and farms, are presented in table 1. At any time, no detectable amount of β-HCH was observed in the case of samples collected from farm C. Milk samples from farm A and farm B were contaminated by β-HCH, although, only in few cases β-HCH levels exceeded the EU limit (0.0030 mg β-HCH/kg). Moreover, for farms A and B, averages and data dispersion, calculated on the basis of the whole period of study, were comparable. Farm D showed more problematic condition, whereas a generalised high milk contamination was observed in farm E.

Table 1 – Milk contamination (means ± SD) by β-HCH (μg β-HCH/kg).

Sampling date	Farm A (n=3)‡	Farm B (n=3)‡	Farm C (n=9)‡	Farm D (n=9)‡	Farm E (n=9)‡
27/07/05-04/08/05	0.0033±0.0029	0.0019±0.0032	nd¥	0.0055±0.0108	0,0440±0.0376
18/08/05	0.0013±0.0004	0.0011±0.0002	nd	0.0009±0.0006	0.0708±0.0922
09/09/05	0.0010±0.0007	0.0008±0.0014	nd	0.0023±0.0035	0.1069±0.0739
29/09/05	0.0018±0.0003	0.0035±0.0006	nd	0.0022±0.0011	0.2845±0.1867
Overall mean	0.0020±0.0015	0.0018±0.0019	-	0.0027±0.0054	0.1266±0.1431

‡number of subjects
¥nd = not detectable (< LOD).

Within each farm and sampling date, the inter-individual variability of milk β-HCH was high with a SDR ranging from 16,7% (Farm A, 29/09/05) to near 200% (Farm D, 04/08/05). Our findings agree with that reported by other authors about milk HCH contamination among

farms (Smit, 1988; Battu *et al.*, (1989) and for the inter-individual variability (Van den Hoek *et al.*, 1975; Smit, 1988, Sitarska *et al.* 1995).

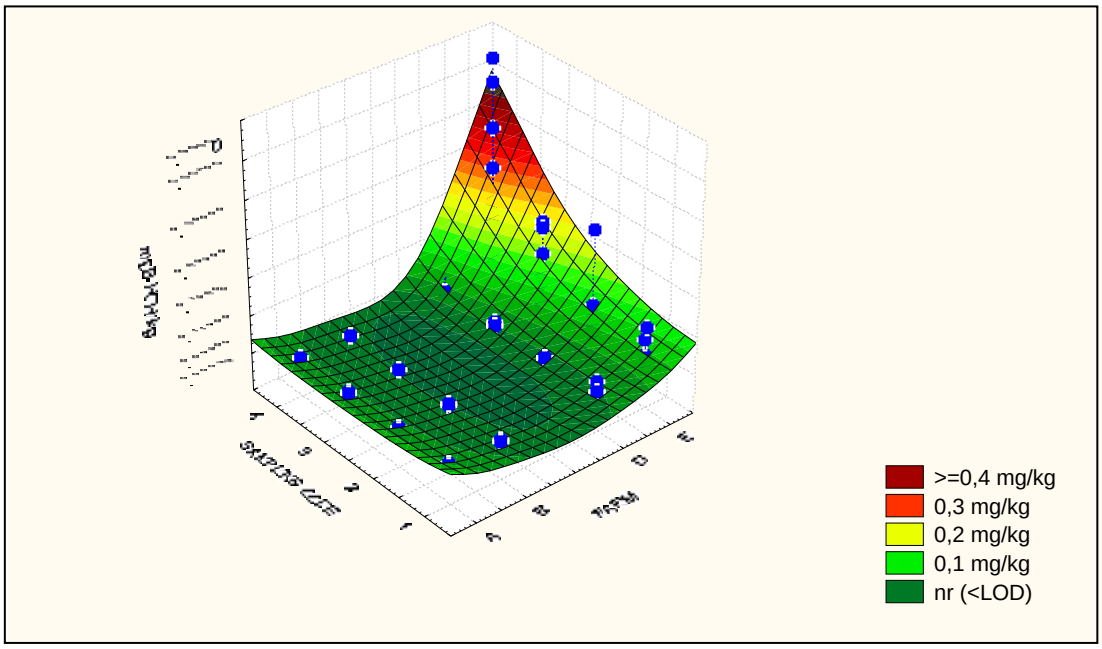

Figure 2 – Milk samples plotted against farm of provenance and date of sampling. Date code: 1=27/07/05-04/08/05; 2=18/08/05; 3=09/09/05; 4=29/09/05.

Figure 2 shows the interaction between sampling time and farms. In particular, it is evident the difference between farm E and the others and between sampling dates: the highest milk contamination was observed at the end of the study. To test the differences a Nested Design ANOVA was performed on standardised data considering also parity and lactating phase as (nested) factors. (Tab. 2).

Table 2 – Nested ANOVA on for milk

Factor	SS	df	MS	F	P
Sampling date[1]	*5,745*	*3*	*1,915*	*4,946*	*0,0038*
Dairy farm (nested in sampling date)[2]	*13,146*	*9*	*1,461*	*3,772*	*0,0008*
N. of lactations (nested in farm)[3]	*1,543*	*1*	*1,543*	*3,985*	*0,0502*
Lactation phase (nested in parity)[4]	*5,267*	*4*	*1,317*	*3,401*	*0,0140*
Error term	*24,394*	*63*	*0,387*	-	-

[1] *Four levels. Date codes: 1=27/07/05-04/08/05; 2=18/08/05; 3=09/09/05; 4=29/09/05*
[2] *Four levels. Farms A, B, D and E. Farm C was not included in the analysis due to lacking of positive cases.*
[3] *Two levels. 1= lactations number ≤2; 2=lactation number >2.*
[4] *Three levels. 1= 0-100 days of the cattle lactation curve; 2=100-200 days of the cattle lactation curve; 3=200-300 days of the cattle lactation curve.*

Date of sampling showed a strong effect (Fig. 3) with a generalised increase of contamination during the course of the study ($P < 0.01$).

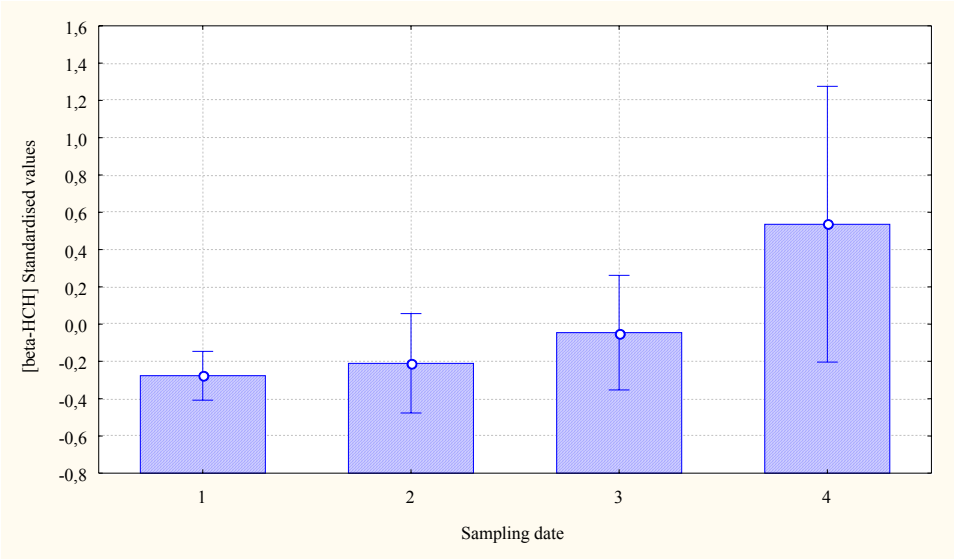

Figure 3 – LS Means of β-HCH concentration in milk (standardized values). Current effect Sampling date: F(3, 63) = 4.9461, p = 0.00380. Type III decomposition. Vertical bars denote 0.95 confidence intervals. Date codes: 1= 27/07/05-04/08/05; 2 = 18/08/05; 3 = 09/09/05; 4 = 19/09/05.

Few information are available in literature about temporal variation of OCP residues in bovine milk. Moreover, no specific research focusing on β-HCH levels in bovine milk, HCH residues in agro-environment and climatological factors has been found in literature. Jhon *et al.* (2001), during a four year study suggests that seasonal climatic conditions may affect the level of milk contamination by OCP, including HCH, in bovine and buffalo milk from Jaipur City (Rajasthan, India). Those authors found a general increase in residues in milk during the winter season when wind, heat and rain are very low in Rajasthan region. They reported that these climatic conditions may affect environmental contamination, reducing the spreading of HCH after pesticide applications. Our and Laquet *et al.* (1974) findings suggest an opposite situation. Laquet *et al.* (1974), in a four years study, found higher concentration of HCH (isomers α+β+γ) in bovine milk during fall and spring than during summer or winter seasons. In particular their study showed a minimum HCH contamination of milk during August 1971 and 1972 and an increase in HCH levels in September and October (around +40% in 1971 and +90% in 1972). A similar behaviour was observed for the heptachlor-epoxide, in 1971, 1972 and 1973.

Temperature and rainfall for the study area, are reported in figure 4. From July to September, rainfall increased (from 0.0 to 41.0 mm of rain per decade) and air temperature decreased (from 35.5°C to 25.4°C as mean of 10 daily peak temperature). These changes indicate that during the experimental period, local climatic conditions could have reduced the HCH isomers migration (Mackay 1991). To support this hypothesis, several studies have showed that HCH isomers migration among different environmental matrices, is primarily affected by air temperature (Komp & McLachlan, 1997; Kelly & Gobas, 2003), wind speed and direction (Glotfelty *et al.*, 1984, Cleemann *et al.*, 1995; Wittich & Siebers, 2002), air humidity and rainfall (Samuel & Pillai, 1990; Hippelein & MacLachlan, 2000).

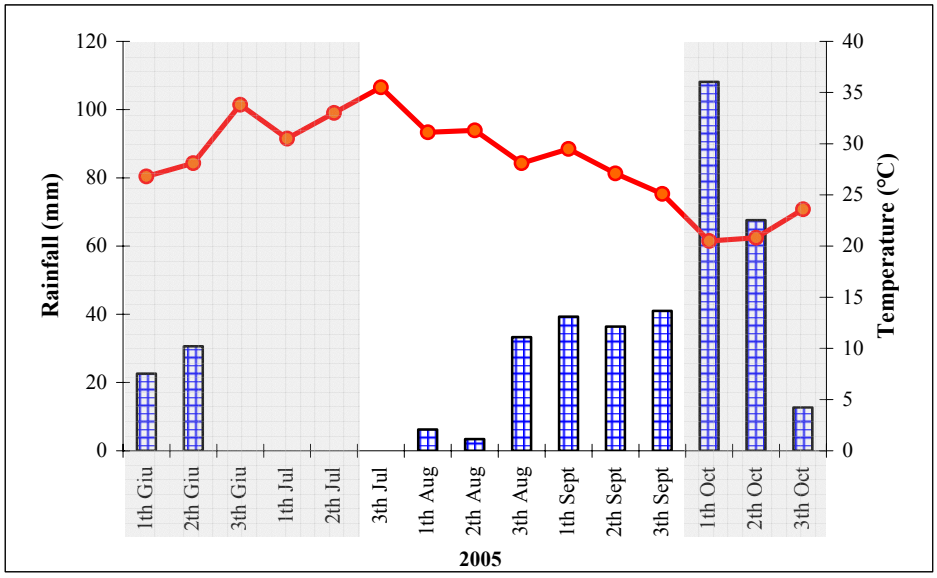

Figure 4 – Means values per decade of air temperature (solid line) and rainfall (vertical bars) recorded at the A.R.S.I.A.L. Anagni Meteorological Station.

A view including the dairy farm as a motive factor, is given in Fig. 5 where data have been grouped on the basis of dairy farms within the sampling dates. Differences were found among dairy farms ($P < 0.001$). Farm E showed a higher milk contamination than farms A, B and D.

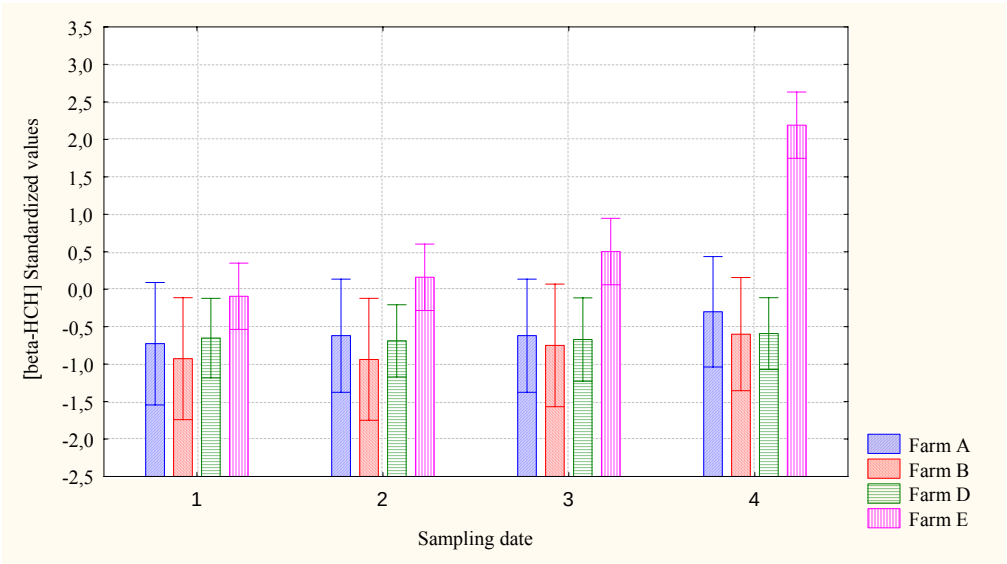

Figure 5 – LS Means of β-HCH concentration in milk (standardized values). Current effect Dairy Farm (Sampling date): F(9, 63)=3.7725, p=0.00076. Type III decomposition. Vertical bars denote 0.95 confidence intervals. Date codes: 1= 27/07/05-04/08/05; 2 = 18/08/05; 3 = 09/09/05; 4 = 19/09/05.

Differences in milk contamination by β-HCH depending on the lactation phase and parity are showed in Fig. 6. Lactation phase and parity affected ($P < 0.05$) milk contamination.

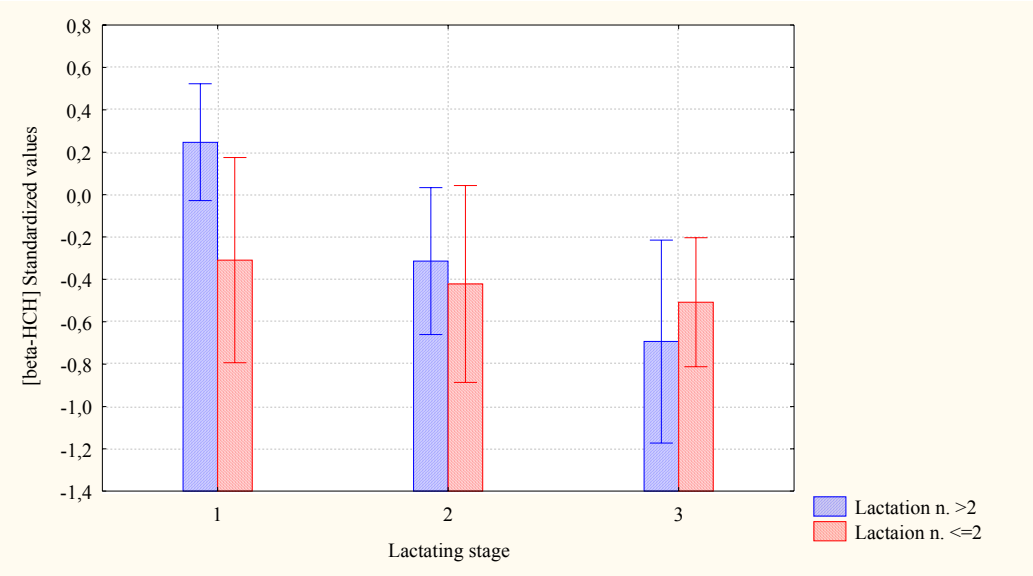

Figure 6 – LS Means of β-HCH concentration in milk (standardized values). Current effect Lactating Stage(Number of Lactations): F(4, 63) = 3.4006, p = 0.01395. Type III decomposition. Vertical bars denote 0.95 confidence intervals. Lactating stage codes: 1= 0-100 days of the cattle lactation curve; 2=100-200 days of the cattle lactation curve; 3=200-300 days of the cattle lactation curve.

Levels of β-HCH in milk were found to be higher during the first 100 days in milk than in the second and third phase of lactation. This was especially evident in cattle with three or more lactations. These findings seems to correlate with the model commonly used to describe milk production in cattle (Wood, 1967; Wood, 1977). The Wood model is able to predict a maximum daily milk production roughly between the 6th and 10th week of lactation. From the peak time, the daily milk production decreases continuously until the end of lactation (conventionally at 305th day). From a physiologically point of view, the higher levels of β-HCH found in first lactating cows agree with that reported by Fries (1977). This author pointed out that the concentrating effect of lipophilic contaminants in milk due to a weight loss is greater than the effect of elimination through milk. Moreover, this fact appear more important in dairy cows at 2th or more lactations than the growing ones for which the dilution effect of lipophilic contaminants may reduce the amount of β-HCH excreted with milk (Fries, 1977; Dixon *et al.*, 2000).

Blood serum contamination by β-HCH

Blood samples collected of eight cows from farm E during the first sampling in august 2005 and the last two samplings in September 2005, were all positive to β-HCH (Tab. 3). As reported above, see table 1, this farm showed high degree of milk contamination by β-HCH. The average level reached by this contaminant in blood, surprisingly decreased from the beginning to the end of the experimental period. In contrast, β-HCH level in milk increased. This may be the consequence of a severe deviation from equilibrium in β-HCH partitioning among fat depots in animal adipose tissue, serum and milk (Waliszewski *et al.* 2004). Such hypothesis might be supported by the poor body condition of dairy cows observed in the last sampling. However considering medians, no more such differences of β-HCH milk content were observed (Tab. 3) suggesting that the average trend may be due to a distribution far away from normality. A quite high degree of variability in blood serum β-HCH level is indicated by the values of standard deviation (coefficient of variation ranged from 43% to 134%). Such heterogeneity was reported in other researches mainly focused on humans

chronically exposed to HCH (Otero *et al.*, 1997; Waliszewski *et al.*, 2000; Karnaus *et al.*, 2005; Waliszewski *et al.*, 2004; Mathur *et al.*, 2005; Thomas *et al.*, 2005).

Table 3 – Descriptive statistics of β-HCH contamination of blood serum samples collected in Farm E.

Sampling date	N	Positive cases	Mean*	S.D.*	SDR	Range*	Median*	25th*	75th*
04/08/05	*8*	*88%*	*0,0329*	*0,0443*	*134%*	*0,1160*	*0,0110*	*0,0012*	*0,0610*
09/09/05	*8*	*100%*	*0,0141*	*0,0151*	*107%*	*0,0491*	*0,0105*	*0,0055*	*0,0160*
29/09/05	*8*	*100%*	*0,0115*	*0,0049*	*43%*	*0,0164*	*0,0120*	*0,0095*	*0,0145*
Overall mean	*24*		*0,0194*	*0,0282*	*142%*	*0,1162*	*0,0110*	*0,0025*	*0,0170*

** data expressed as µgβ-HCH/ml*

The relationship between blood serum levels of β-HCH and milk is reported in Fig. 7a,b. Using all data there was no relationship (r^2 = 0.0124, P = 0.446) between blood vs. milk (Fig. 7a). Excluding two cases falling outside the 95% confidence limit of regression the relationship (r^2 = 0.8519, P = 0.009) becomes significant (Fig 7b). Moreover, such an exclusion of these outlier cases, leaded to a not significance of the intercept value (P = 0.065) adding robustness to the linear relation.

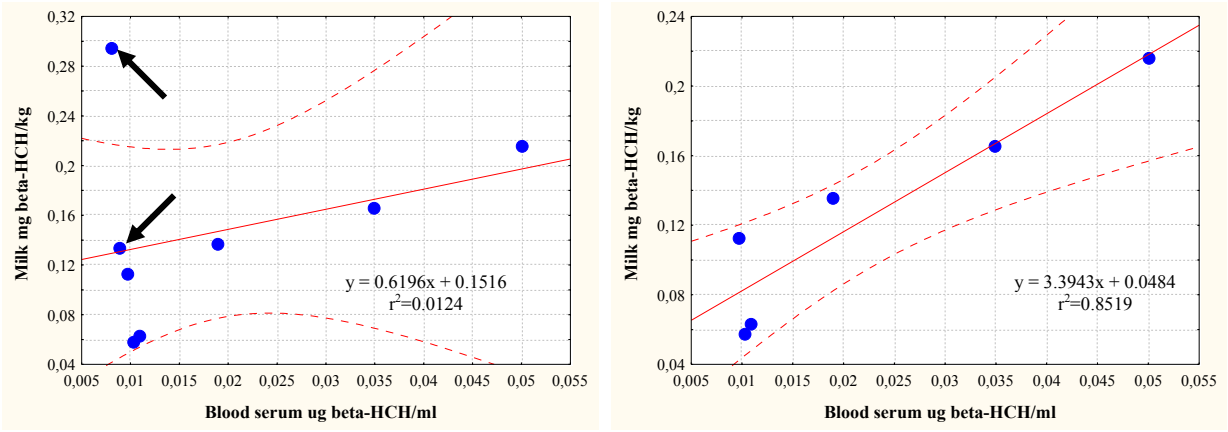

Figura 7 – Farm E, mean β-HCH level found in blood serum Vs milk content from eight dairy cows (a) and only six (b). Two cases indicated in a) by an arrow was outside of the 95% confidence limits (dashed red lines) and was treated as outliers in b).

As reported above, cows from farm C were always negative for β-HCH in milk throughout the study. Three blood samples, were analysed to determine β-HCH as a negative control. In two cases a trace (below the LOQ) of β-HCH were found (estimated level 0.0003 µg β-HCH/ml). As pointed out by several authors (Gupta *et al.*, 1978; Murphy & Harvey, 1985; Kokan *et al.*, 1994; To-Figueras *et al.*, 1997; Otero *et al.*, 1999; Waliszewski *et al.*, 2004) our results suggests an high sensibility in using the blood burden as early indicator of OCP exposure in dairy cattle and potential impact on human well-being.

CONCLUSIONS

Preliminary results about a study case of agro-environmental contamination by HCH in the Sacco river floodplain happened in 2005, are reported. Four dairy farms of the five controlled were positive to β-HCH in milk. Milk contamination changed during the course of the samplings and among farms. It is conceivable that meteorological parameters, soil and forages contamination and herd managing practices play an important role in β-HCH levels found in milk samples.

The degree of β-HCH excretion was found higher in animals in the first phase of lactation than in the remnant ones, whereas parity does not seem to have any effects.

In spite of the elevated variability of β-HCH levels, a satisfactory relationship was found between milk and blood contamination. Traces of β-HCH were found in blood of animals negative for β-HCH contamination of milk, suggesting an interesting application of blood serum as early marker of cattle exposure and agro-environmental contamination by HCH.

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