THE ITALIAN BUFFALO MILK CASE – RESULTS AND DISCUSSION OF PCDD/F- AND DL-PCB ANALYSIS IN MILK, FEEDING STUFF AND SOIL SAMPLES FROM CAMPANIA, ITALY

Neugebauer, Frank[1], Esposito, Mauro[2], Opel, Matthias[1], Päpke, Olaf[1], Gallo Pasquale[2], Cavallo Stefania[3], Colarusso Germana[3], Rosa D'Ambrosio[3], Sarnelli Paolo[4], Baldi Loredana[2,3], Iovane Giuseppe[2], Serpe Luigi[2]

[1]Eurofins | GfA, Neuländer Kamp 1, D-21079 Hamburg, Germany; [2]Istituto Zooprofilattico Sperimentale del Mezzogiorno, Via della Salute,2 80055 Portici, Italy; [3]O.R.S.A. Campania, Via della Salute, 2 80055 Portici, Italy; [4]Regione Campania – Settore Veterinario, Centro Direzionale isola C/3, 80143 Napoli, Italy; e-mail: FrankNeugebauer@eurofins.de

Introduction

The finding of elevated concentrations of polychlorinated dibenzo-p-dioxins and –furans (PCDD/F) and dioxin-like PCB (dl-PCB, "WHO-PCB") in milk, especially buffalo milk of the district of Caserta in the northern part of the rural region of Campania, Italy[1,2,3] lead to a broad discussion in the last years with regard to concerns about human health in connection with the consumption of Mozzarella, a regional cheese specialty produced from buffalo milk.

Extensive monitoring programmes have been set up in order to control the situation and examine the contamination extent and sources. The programmes have been especially intensified in 2008, when over 1200 samples have been drawn and analysed, mainly from buffalo milk and other milk. Also related samples, such as feeding stuff from the farms concerned and local soil samples were analysed in order to trace back the contamination to its sources. A long history of illegal local waste burning throughout the whole region is supposed to be responsible for the dioxin contamination[3]. This assumption can be supported by the present data which were generated by High Resolution Mass Spectrometry (HRMS) analyses of PCDD/F and dl-PCB. This technique with its richness in additional information together with the high number of data sets gave a reasonably sound discussion base and allowed for considerations such as source identification for finding the contamination source as well as to link findings in milk, feeding stuff and soil.

Materials and Methods

The samples were taken from over 600 farms in Campania, Italy, mainly in the districts of Caserta and Napoli (Naples). They consist of buffalo milk and other milk as well as soil samples and different types of animal feed samples, being analysed for PCDD/F and dl-PCB. Project management and sampling has been conducted by regional authorities (Istituto Zooprofilattico Sperimentale di Mezzogiorno (IZSM) Portici; O.R.S.A. Campania and the ARPAC that are the responsible institutes for food safety and environmental safety in the region of Campania). The analysis of the samples was performed by the Eurofins GfA GmbH laboratory Hamburg, Germany using a HRMS method according to EU legislation. Milk samples were extracted by sodium oxalate assisted liquid/liquid extraction, feeding stuff and soil samples were dried, grinded and extracted using Soxhlet extraction with toluene. The sample clean-up consisted of a multi-step column chromatography. Analysis was performed on Waters AutoSpec mass spectrometers at a mass resolution of R ≥10000 by isotope dilution with every analysed compound (exception: 123789-HxCDD) having its own $^{13}C_{12}$-labelled internal quantification standard added to the sample before extraction. The overall analytical quality has been accompanied by a QA/QC-scheme with laboratory blanks as well as control analyses of reference materials. Analytical data have been grouped, based upon the original sample description as present in the IZSM institute.

Feeding stuff samples have been divided into two groups one of which consisted of forages being plant feed such as silage, grass and hay of mainly local origin[3] which are consumed by the buffalos as main nutrition. The second group, referred to as "other feed", consists of all other types of animal feed, e.g. premixes, mixed feed, unifeed, supplements etc. As a consequence, the comparison against EU limit values has been restricted to the EU limits of the "plant feed" and "compound feed" categories.

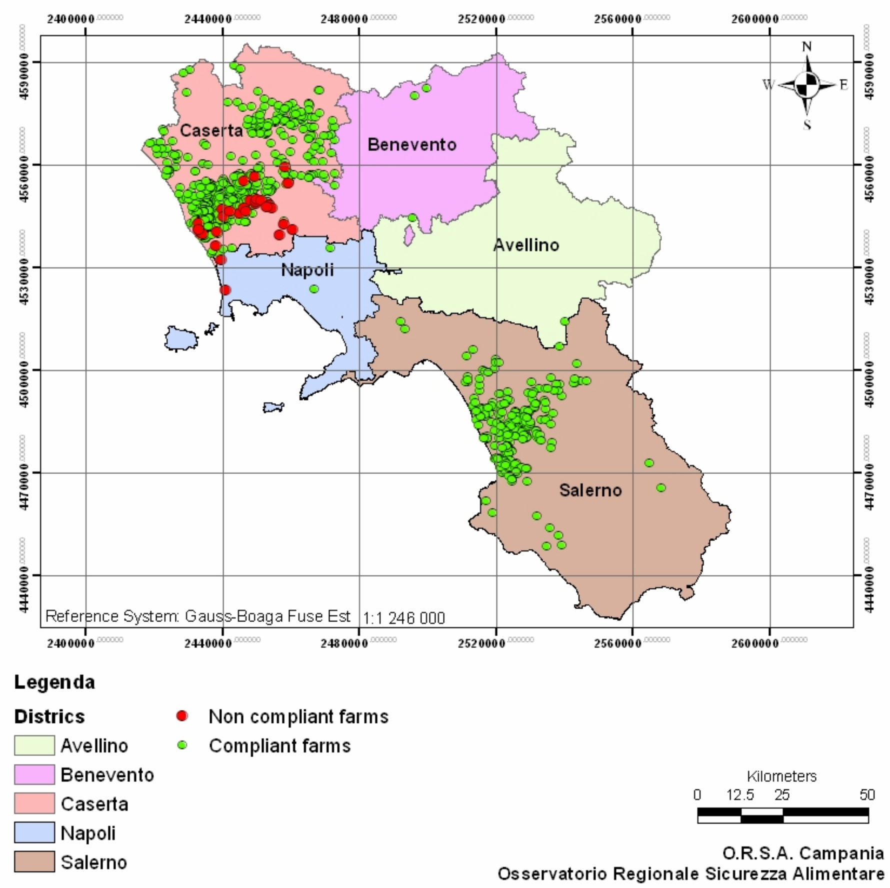
Results

A total of 1198 samples of different samples from Campania, analysed between August 2008 and March 2009 have been included in this study. From these samples, 987 originate from Caserta district, where the main contamination of buffalo milk occurred (*table 1*). The others come from the neighbouring district of Napoli (Naples) and from the generally not contaminated districts of Salerno, Benevento and Avellino (*figure 1*).

Table 1: Total sample numbers of the analysed samples

	total	sample numbers					
		buffalo milk	cow milk	sheep milk	forages	other feed	soil
all samples	1198	732	57	20	164	61	164
whereof from Caserta	987	555	48	10	161	61	152

Figure 1: map of Campania region showing the sampling points (farms) for buffalo milk



The overall statistics of the samples are given below (*tables 2,3*), also presenting the results for Caserta only, because these data were taken for PCDD/F-pattern discussion. <u>All quoted TEQ-values are presented as maximum (upper-bound) WHO-TEQ (1998), for direct comparability also used for soil</u>. Calculation base is pg/g fat for milk, wet weight calculated to 12 % moisture for feeding stuff and dry weight for soil. The results show a considerable number of samples above legislative limits with sheep milk giving the highest concentrations, exceeding EU maximum limits in about 50% of the cases, whereas the other milk samples would give lower average results with buffalo milk exceeding the limits in 27% of the samples (cow: 18%) for PCDD/F. These concentration levels and also the observed relations are in consistence with previous survey programs performed in the region[1,3]. Regarding Caserta only, these percentages are much higher.

Table 2: PCDD/F results (upper-bound WHO(1998)-TEQ)

PCDD/F-TEQ (WHO1998), incl.	total						Caserta					
	buffalo milk	cow milk	sheep milk	forages	other feed	soil (+)	buffalo milk	cow milk	sheep milk	forages	other feed	soil (+)
	pg/g fat	pg/g fat	pg/g fat	ng/kg 12% w.w.		ng/kg dm	pg/g fat	pg/g fat	pg/g fat	ng/kg 12% w.w.		ng/kg dm
average	2,89	1,80	4,25	1,06	0,17	1,49	3,30	1,96	6,62	1,07	0,17	1,40
median	1,41	1,07	2,79	0,17	0,10	1,05	1,65	1,27	5,86	0,17	0,10	1,05
min	0,21	0,39	0,28	0,05	0,05	0,89	0,21	0,54	0,84	0,05	0,05	0,89
max	87,00	8,98	12,90	60,40	0,76	12,80	87,00	8,98	12,90	60,40	0,76	12,30
EU action limit (*)	2	2	2	0,5	0,5	n/a	2	2	2	0,5	0,5	n/a
% > EU action limits	38,8%	22,8%	55,0%	22,0%	9,8%	n/a	44,5%	25,0%	90,0%	21,7%	9,8%	n/a
EU maximum limit (*)	3	3	3	0,75	0,75	n/a	3	3	3	0,75	0,75	n/a
% > EU maximum limits	26,6%	17,5%	50,0%	18,3%	1,6%	n/a	31,5%	20,8%	80,0%	18,0%	1,6%	n/a

Table 3: PCB results (upper-bound WHO(1998)-TEQ)

PCB-TEQ (WHO1998), incl.	total						Caserta					
	buffalo milk	cow milk	sheep milk	forages	other feed	soil (+)	buffalo milk	cow milk	sheep milk	forages	other feed	soil (+)
	pg/g fat	pg/g fat	pg/g fat	ng/kg 12% w.w.		ng/kg dm	pg/g fat	pg/g fat	pg/g fat	ng/kg 12% w.w.		ng/kg dm
average	1,39	1,46	2,49	0,31	0,07	0,26	1,56	1,57	3,82	0,31	0,07	0,25
median	0,90	1,24	1,71	0,09	0,05	0,19	0,99	1,30	4,34	0,09	0,05	0,20
min	0,13	0,56	0,48	0,03	0,03	0,09	0,21	0,59	0,93	0,03	0,03	0,09
max	15,90	3,29	8,40	15,90	0,26	1,97	15,90	3,29	8,40	15,90	0,26	1,97
EU action limit (*)	2	2	2	0,35	0,5	n/a	2	2	2	0,35	0,5	n/a
% > EU action limits	19,7%	22,8%	50,0%	13,4%	0,0%	n/a	24,1%	27,1%	80,0%	13,7%	0,0%	n/a

(+) calculated as upper-bound WHO(1998)-TEQ for reason of direct comparability;

(*) EU limits[4,5,6] read as "action levels/maximum levels" for food and as "action threshold/maximum contents" for feeding stuff. For feeding stuff of the category "forages", the EU limits for feed of plant origin is the reference, for the category "other feed", the EU limits for "compound feed" have been used, since further details are partially unknown for the individual samples.

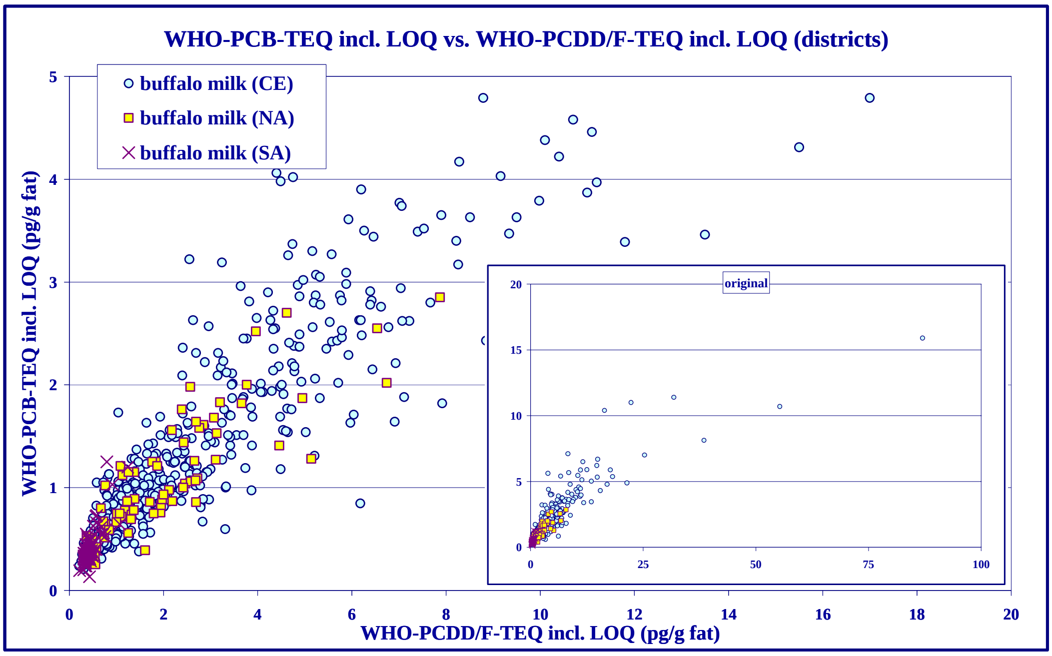
The TEQ-contributions resulting from dl-PCB and PCDD/F can be seen in *table 4*. PCDD/F accounts for the major part of the contamination, whereas the dl-PCB give a contribution of about 30-45% of the total TEQ, with exception of soil having a significantly lower contribution of dl-PCB with lower values for other feed and soil.

Table 4: average contributions of dl-PCB and PCDD/F towards the total TEQ values

	buffalo milk	cow milk	sheep milk	forages	other feed	soil
PCDD/F-TEQ	67,5%	55,2%	63,0%	77,3%	70,7%	85,4%
PCB-TEQ	32,5%	44,8%	37,0%	22,7%	29,3%	14,6%
whereof: co-PCB	*27,9%*	*40,5%*	*33,5%*	*21,7%*	*26,6%*	*12,8%*
whereof: mo-PCB	*4,6%*	*4,3%*	*3,5%*	*0,9%*	*2,7%*	*1,8%*

One important fact can be seen from a plot of the upper-bound TEQs of dl-PCB vs. PCDD/F for the buffalo milk samples, having a reasonable overall correlation between the two groups (*Figure 2*) extending over the whole range of results. For the higher contaminated districts it does not show apparent sub-groups, pointing towards a homogeneous data set. Anyway, two effects can be seen. One is an increased scattering at the extreme upper end of the concentrations and the other is a distortion at the lower end, where the influence of the non-detected compounds can be seen, contributing more and more towards upper-bound TEQ-values. The present data are in good accordance with an evaluation of PCDD/F- and dl-PCB data from another study[2].

Figure 2: zoomed plot of dl-PCB-TEQ against PCDD/F-TEQ (original graph inside). Abbreviations used for the districts of Campania: CE=Caserta, NA=Napoli, SA=Salerno)



<u>*PCDD/F Pattern recognition for samples from Caserta*</u>

HRMS is a technique delivering the possibility of pattern recognition and therefore source discussion by comparing the relations of the analysed parameters[7]. Even by only analysing the 2,3,7,8-substituted PCDD/F, thus only generating a minor part of the pattern information, some conclusions can be drawn. The data used has been restricted to samples from the Caserta district in order to focus onto the mainly contaminated area, using findings above LOQ only. Discussing PCDD/F contents, the relation between the total dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF) – in this case the ratio between the 2,3,7,8-substituted ones – is valuable for the discussion (*table 5*). Please note that we are discussing original concentration shares here, not TEQ shares.

Table 5: PCDF- / PCDD-distribution (total of 2,3,7,8-substituted congeners) for samples from Caserta district

distribution of PCDF : PCDD (totals of 2,3,7,8-substituted congeners), samples from Caserta region						
	buffalo milk	cow milk	sheep milk	forages	other feed	soil
total 2,3,7,8-PCDD	36,5%	40,4%	41,0%	42,8%	76,0%	81,2%
total 2,3,7,8-PCDF	63,5%	59,6%	59,0%	57,2%	24,0%	18,8%

There is a good correlation between PCDD and PCDF for all milk sample groups and different correlations for the feeding stuffs and the soil. It is a first indication that the PCDD/F pattern would be uniform, giving another confirmation of the general data homogeneity. Generally the PCDF are present to a higher extent than the PCDD which might indicate influences from combustion and/or additionally PCB as a contamination source[8].

For a more detailed evaluation, suitable average patterns have been deduced by using only data sets from higher contaminated samples, i.e. above the respective EU action limits and soil above 2 ng WHO-TEQ/kg. This also helps to exclude background effects. The results are given in *figures 3 and 4*, normalised for highest congeners.

Figure 3: average PCDD/F-pattern for buffalo milk (n=247) and cow milk (n=12), normalised (*)

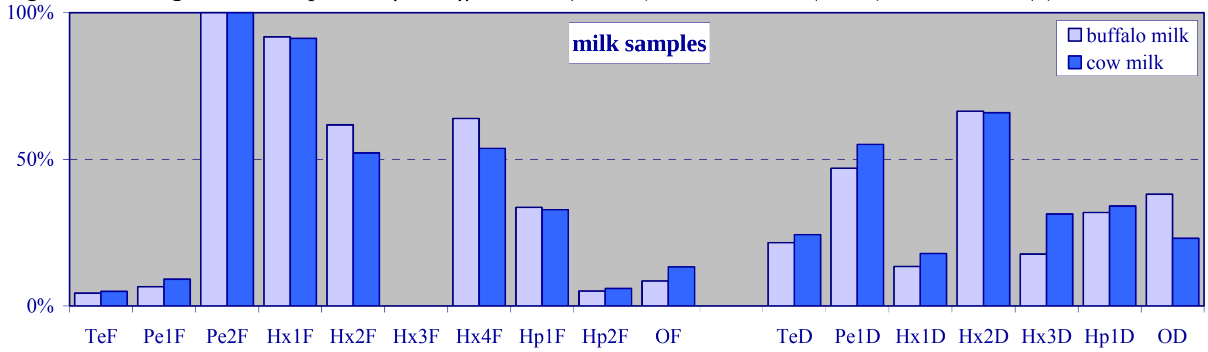
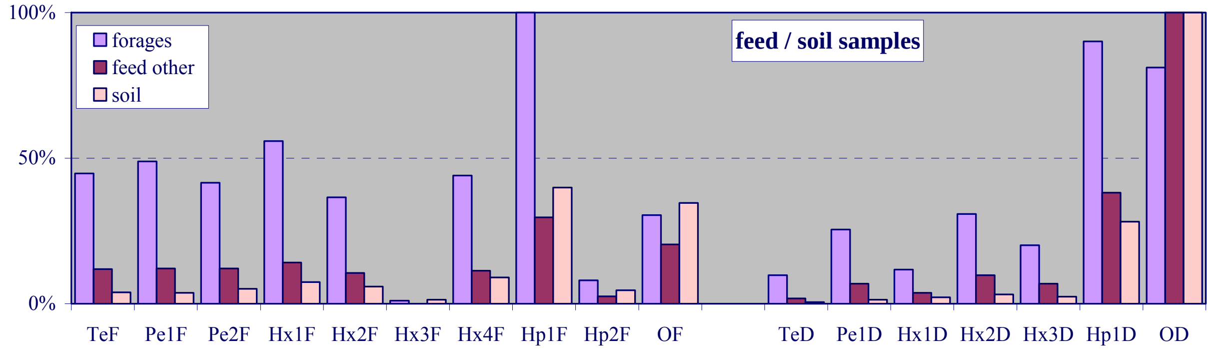


Figure 4: average PCDD/F-pattern for forages (n=35), other feed (n=6) and soil (n=14), normalised (*)



(*) to congener with highest concentration; abbreviations: Te=2378-Tetra; Pe1=12378-Penta; Pe2=23478-Penta; Hx1=123478-Hexa; Hx2=123678-Hexa; Hx3=123789-Hexa; Hx4=234678-Hexa; Hp1=1234678-Hepta; Hp2=1234789-Hepta; OF=OctaCDF; OD=OctaCDD

Conclusions

The present contamination of buffalo milk is mainly caused by PCDD/F, which contribute to about two thirds to TEQ values, whereas dl-PCB contribute only one third. Nevertheless there is a significant contribution derived from dl-PCB. The percentage of samples being above the European legislative limits at around 27% of the buffalo milk samples for PCDD/F-TEQ is in the same order of magnitude as in the last years[2,3].
The contamination of feeding stuff sampled together with the milk samples points towards forages such as grass for being the main reason for the found PCDD/F- and dl-PCB levels in milk (for cow milk cfr. McLachlan[9]). This is also confirmed by the fact that the overstepping of the EU maximum contents for animal feed are almost exclusively found in the forages which consists of local silage, hay and grass. There, 18% of the samples exceed the EU-PCDD/F limit, whereas only about 2% of the other feed samples exceed the limits.

Considering the relative impact of soil contamination, by far the the greatest influence on cow milk originates from forages[9]. For buffalos anyway, it is worth taking a closer look, since the daily intake of soil can be at about 5-20% of the daily intake of dry feed. For a very rough estimation we start from the median of the single PCDD/F-concentrations incl. LOQ in soil from Caserta (equal to 1.05 ng WHO-TEQ/kg). Assuming a daily soil intake of 0.8 kg/d per animal (5% of 16kg feed), a daily milk production of 10 kg/d at 8.5% fat and some recent carry-over rates for cows[10] we will end up with about 0.5 ng PCDD/F-TEQ/kg milk fat which is at 25% of the EU limit for milk. With a soil concentration of 10ng WHO-TEQ/kg the result of this calculation exceeds the EU limit by a factor of 2.5!

As for PCDD/F patterns, the congener distribution of the major part of the soil and feeding stuff samples points toward a combustion pattern, having e.g. the 12378- and 23478-PentaCDF or the 123478-, 123678- and 234678-HexaCDF at the same concentration levels. The higher level of 123478-HxCDF indicates a certain but not exclusive influence of PCB or PCB-related combustion. There is no extremely high difference between the two

as would be the case with a pure PCB pattern. Also, an exclusive and direct impact from PCB can be ruled out because of the significant contribution of PCDD towards the total Dioxin TEQ and the ratio of PCDF to PCDD. Considering other possible sources, there is no hint for some of the specific PCDD/F-patterns originating from "chemical production sources", i.e. no indication for PCDD/F-patterns of Chlorophenol-(PCP-) or electrolysis-type. This is concluded from the absence of excessively high amount of OctaCDD/F as well as from the absence of an extremely high differences e.g. between the Penta- to HeptaCDF congeners or an especially high 2378-TetraCDD as compared to the other PCDD congeners.
A comparison between soil/feed samples and milk samples is only partially possible because of the metabolisation effects. The behaviour of buffalo and cow milk is similar with respect to the relation of PCDD/F and PCB, the ratio of PCDF vs. PCDD and the typically strong depletion of certain congeners. That allows us to assume a strong similarity in PCDD/F-metabolism for these two species and therefore to use carry-over rates (COR) established for cows[9,10,11] also for buffalo milk. A direct comparison is possible only for the relative ratio of the hexachlorinated compounds, since they would behave similar throughout metabolisation. That the milk samples as well as soil/feed show similar ratios especially for the HexaCDD/F, points towards the direct link between them.
PCDD/F and PCB are two different groups of chemicals which are generally not directly linked to each other. The only exception is the technical PCB mixtures that also contain PCDD/F as unintended by-products. The technical PCB can not be considered the main cause for the present contamination, because this would require much higher PCB findings in feed and soil as well as it would give a contribution of dl-PCB towards the total TEQ values which is by far higher. Taking this into consideration, and seeing the correlation between dl-PCB and PCDD/F found together in the local buffalo milk samples, the most likely assumption for the PCDD/F- and PCB findings is a variety of wastes containing some PCB being commonly transferred by atmospheric transport after combustion, which formed a combustion pattern with respect to PCDD/F as well as PCB**.** Illegal waste deposition has already been proposed as an explanation. The present data are suitable to support this theory.

A more detailed examination, using the advantages of the HRMS technique especially for non-2378-compounds and fingerprint information could further add to this picture. It is recommended to conduct a more in-depth evaluation of present data as well as to collect air monitoring samples in order to examine the exact pathways of regional air transport and to directly pinpoint possible sources. The differences in pattern and contents of PCDD/F and PCB and their influence on the milk contamination can only be discussed by having a mass balance for these compounds around the system "buffalo".

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