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► To cite this version:

Luisa Cigliano, Carlo Nebbia, Guido Rychen, Cyril Feidt, Flavia Girolami, et al.. Evaluation of serum markers of blood redox homeostasis and inflammation in PCB naturally contaminated heifers undergoing decontamination. *Science of the Total Environment*, 2016, 542 (A), pp.653-664. 10.1016/j.scitotenv.2015.10.104 . hal-01575592

HAL Id: hal-01575592

<https://hal.univ-lorraine.fr/hal-01575592>

Submitted on 14 Apr 2022

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Evaluation of serum markers of blood redox homeostasis and inflammation in PCB naturally contaminated heifers undergoing decontamination

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Published in Science of the Total Environment. Available online: <http://dx.doi.org/10.1016/j.scitotenv.2015.10.104>

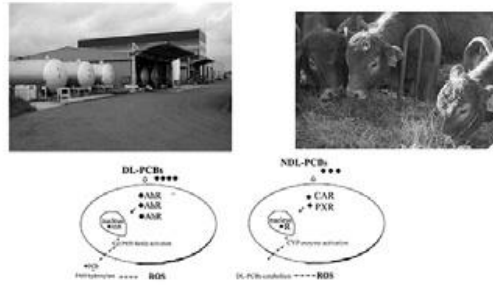
Keywords

Biomarkers, Environmental pollution, PCB decontamination, Redox homeostasis, Haptoglobin, Oxidativestress.

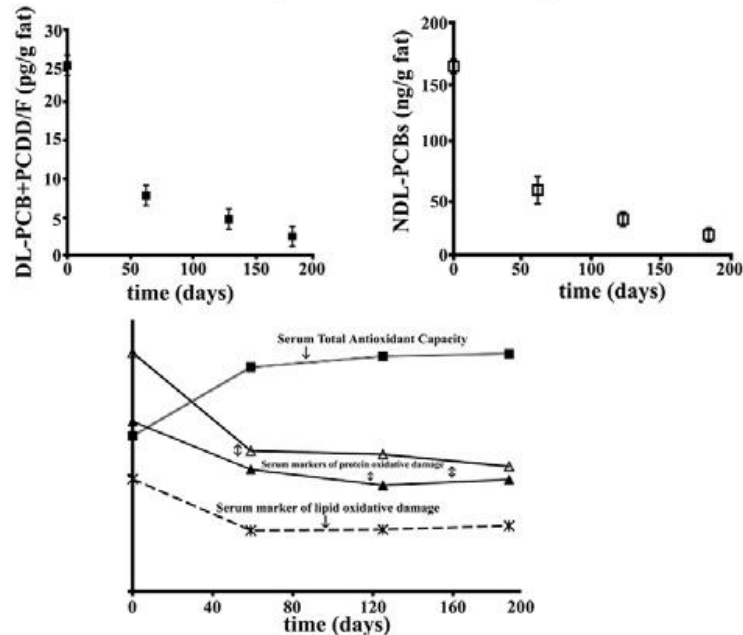
Abstract

Dioxins and polychlorinated biphenyls (PCBs) are widely spread and long persistent contaminants. The aim of this study was to evaluate physiological changes associated with the decontamination of animals previously exposed to environmental pollutants. Eight Limousine heifers were removed from a polluted area and fed a standard ration for six months. The extent of contamination was defined by measuring total toxic equivalents (TEQ) values of dioxin like-PCBs (DL-PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated di-benzofurans (PCDFs), and NDL-PCBs amount in pericaudal fat two weeks after the removal from the contaminated area (day 0) and then bimonthly for six months during the decontamination (days 59,125, and 188). The concentrations of both DL-PCBs + PCDD/Fs and NDL-PCBs at the start of decontamination (day 0) were higher than those legally admitted, and they were strongly decreased at the end of the experimental period. Specific indices of blood redox homeostasis and inflammation were also measured at each time. Serum concentrations of Retinol, Tocopherol and Ascorbate, the total antioxidant capacity (TAC) and the activities of superoxide dismutase and glutathione peroxidase were lower at day 0 than after 59,125 or 188 days of decontamination. Protein-bound carbonyls (PC), nitro-tyrosine (N-Tyr), and lipid hydroperoxides concentrations were higher at day 0 than during decontamination. In addition, TAC, PC and N-Tyr levels correlated with both DL-PCB and NDL-PCB concentrations only at day 0. Serum concentrations of TNF-alpha and Haptoglobin were higher in samples collected at day 0 than in those obtained during decontamination. As Haptoglobin and TNF-alpha levels correlated with both DL-PCB and NDL-PCB concentrations at day 0 and at day 59 (when these concentrations are still over legal limit), they might represent easily measurable parameters for assessing acute exposure to pollutants. Further both N-Tyr and TNF-alpha concentrations could be used as bio-monitoring markers of the decontamination procedure.

Limousine heifers exposed to PCBs by feeding forages contaminated by emissions of an industrial plant.



Decontamination: transfer in an experimental farm and feeding a not contaminated diet



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Introduction

Persistent organic pollutants (POPs) are toxic to human health and ecosystems, and are largely transferred in the food chain (Rychen et al., 2005; Antignac et al., 2006). Several POPs, such as dioxin-like and non-dioxin-like polychlorinated biphenyls (DL-PCBs and NDL-PCBs respectively) are voluntarily produced, for commercial purposes, as a result of industrial activities, while other pollutants, such as polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), designed as dioxins, are unintentional byproducts of industrial processes. Dioxins and PCBs are toxic chemical compounds of great public health concern, and represent the major sources of environmental contamination. Due to their structural stability and volatility are widely spread, thus affecting agricultural areas near or far away from emitting sources (Beyer et al., 2000; Lohman and Seigneur, 2001). They are also resistant towards chemical and biological degradation processes, thus showing a long persistence in the environment. Although the Stockholm Convention on Persistent Organic Pollutants in 2001 banned PCB production internationally (UNEP, 2001), PCBs are still found in soil, fresh water, aquatic wildlife, and mammals, essentially because of the seepage into the environment, accidental spills, and improper disposal. Food chain contamination occurs as a consequence of the animal

ingestion of contaminated water, soil and forage (McLachlan, 1993; Thomas et al., 1999a, 2002; Brambilla et al., 2004; Rychen et al., 2014). Indeed, dioxins and PCBs are lipophilic and accumulate in animal body, mainly in the liver and in the adipose fraction of organs and tissues (Larsen, 2006), being also transferred into milk and eggs. Therefore, the consumption of animal products rich in fat represents by far the major source of exposure for humans (Thomas et al., 1999b; Schecter et al., 2006). However, ruminants were reported to be decontaminated (McLachlan, 1994; Thomas et al., 1999b; Huwe and Smith, 2005; Rossi et al., 2010; Rychen et al., 2014), essentially via milk excretion (Thomas et al., 1999b; Glynn et al., 2009; Rossi et al., 2010), or through the allometric increased volume of adipose tissue during growth (Chamberland et al., 1994; Glynn et al., 2009; Rychen et al., 2014).

Dioxins and DL-PCBs share a common toxicity mechanism, that is mediated via binding to a specific intracellular receptor, the aryl hydrocarbon receptor (AhR) (Alsharif et al., 1994; Mandal, 2005), whose activation is responsible for the enhanced expression of genes coding for cytochrome P450 1 family enzymes in liver of several species, including cattle (Safe, 1986; Whitlock, 1990; Machala et al., 1998; Matsumura, 2003; Guruge et al., 2009).

In particular, PCB 126, the most potent AhR agonist among DL-PCBs (Bandiera et al., 1982), not only alters the expression of genes coding for CYP1A1, but also impairs the expression of genes coding for antioxidant enzymes, resulting in oxidative stress in the liver (Hassoun et al., 2002; Parkinson et al., 1983). Dioxin exposure was reported to promote, via AhR activation, highly reactive oxygen species (ROS) production (Slezak et al., 2000; Nebert et al., 2000; Dalton et al., 2002), and to depress several ROS quenching systems (Ishida et al., 2009), thus inducing increased DNA fragmentation, as well as production of superoxide anion, thiobarbituric acid reactive substances, and hydroperoxides (Shertzer et al., 1995; Zhao and Ramos, 1998; Slezak et al., 2000, 2002). This leads to oxidative conditions (Shertzer et al., 1998; Slezak et al., 2000; Senft et al., 2002) that may induce oxidative stress-related processes (Mandal, 2005; Pelclova et al., 2011), that are associated with modifications of physiological and metabolic functions (Halliwell and Gutteridge, 2000). NDL-PCBs, due to their chemical properties, have a low affinity for AhR, and are mainly involved in alterations of signal transduction systems, neurotoxicity, immune suppression and endocrine disruption (Fischer et al., 1998; Selgrade, 2007). NDL-PCB bioaccumulate preferentially in adipose tissue causing disruption of lipid metabolism and induction of IL-6 and TNF- α production (Ferrante et al., 2015). NDL PCBs were also reported to reduce cell viability and induce oxidative stress (Westerink, 2014; Abella et al., 2015).

In physiological conditions, the antioxidant defence system, provided by enzymes and antioxidants, scavenges ROS, thus

limiting or preventing oxidative damage (Halliwell and Gutteridge, 2000). Oxidative stress occurs as consequence of an imbalance between ROS production and neutralizing capacity of antioxidant mechanisms (Halliwell and Gutteridge, 2000), and is involved in the aetiology of several diseases and metabolic disorders (Lomba, 1996; Bernabucci et al., 2002, 2005; Castillo et al., 2005; Wilde, 2006), also contributing to the reduction of fertility in dairy cows (Wathes et al., 2012). Therefore, the evaluation of blood redox homeostasis has increasingly contributed to knowledge of the processes involved in reproductive and metabolic disorders (Campbell and Miller, 1998; Kankofer, 2002; Sordillo and Aitken, 2009), and it has become important as a complementary tool for the evaluation of health and metabolic status of dairy cows (Bernabucci et al., 2005; Castillo et al., 2005, 2006).

The main objective of this investigation was to evaluate the effect of a decontamination procedure, based on the removal of Limousine heifers from an agricultural area contaminated by emissions of an industrial plant specialized in PCB treatment, followed by the feeding a standard ration, on specific indices of blood redox homeostasis and inflammation, in order to assess the changes associated with decontamination, and to obtain tools for monitoring such a process. We focused on the analysis of oxidative and inflammatory status essentially because both DL-PCBs and NDL-PCBs, although through different pathways, are able to disrupt redox homeostasis and to induce inflammatory response.

Material and Methods

Materials

Bovine serum albumin fraction V (BSA), chemicals of the highest purity, Rabbit anti-human Hpt IgG, Goat anti Rabbit IgG-horseradish peroxidase linked (GAR-HRP), Rabbit anti-dinitrophenylhydrazine (anti-DNP) IgGs, and standards for high performance liquid chromatography (HPLC) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The Nucleosil 100-NH₂ column (5 μ m particle size, 250 \times 4.6 mm i.d.) and the Nova-PAK C18 column (4 μ m particle size, 125 \times 2 mm i.d.) were obtained from Macherey-Nagel (Duren, Germany). Organic solvents were purchased from Romil (Cambridge, UK). Polystyrene 96-wells plates were purchased from Nunc (Roskilde, Denmark). Nitrated BSA and the kit for titration of lipoperoxide of Cayman Chemical, as well as rabbit anti-nitrotyrosine IgG of Covalab were purchased by Vincibiochem (Vinci, Italy). The dye reagent for protein titration, recombinant bovine TNF- α , mouse anti-bovine TNF- α IgG, biotinylated anti-TNF- α IgG, and Streptavidin conjugated to HRP were from AbD Serotec (Bio-Rad Laboratories, Hercules, CA).

Farm selection and animals

The study was carried out on eight one year old contaminated Limousine heifers reared in a farm close to an industrial plant (Greze en Bouère, 53, Mayenne, France). During the first year of their life, the animals were exposed to emissions of organic pollutants, particularly DL-PCBs and NDL-PCBs. In detail, the

heifers here studied were reared in an agricultural area near to Aprochim, an industrial plant specialized in treatment and decontamination of equipments, transformers, and oils contaminated by PCBs. The accidental exposure to pollutants was essentially due to the ingestion of forages contaminated by emissions of the factory Aprochim. This pollution was identified as "PCB contamination" because a control performed by the local prefecture revealed that samples of milk, meat, forages and dairy products, collected in 8 farms located within 3 km from the industrial plant, contained a PCB concentration higher than that legally admitted. The commercialization of meat and dairy products from these farms was then forbidden, and in several farms animals were slaughtered. Indications on the PCB contamination around Aprochim are available online (www.leparisien.fr/.../pcb-trois-elevages-bovins-contamines-abattus-en-m, <http://www.vedura.fr/actualite/6945-pollution-pcb-mayenne-troupeaux-abattus-usine-aprochim-mise-demeure>; <http://www.parismatch.com/Actu/Environnement/Usines-Aprochim-pollueur-vainqueur-595816>).

The extent of contamination was evaluated by measuring total toxic equivalent (TEQ) values of DL-PCB + PCDD/F, and NDL-PCB amount in pericaudal fat of each animal. The heifers were removed from the contaminated area (Mayenne, 53) and housed in an experimental facility located far away this area (Meurthe et Moselle, 54, at the "Domaine experimental de la Bouzule", Lorraine University-Ecole Nationale Supérieure d'Agronomie et des Industries Alimentaires; Vandoeuvre, France) for six months. The animals were reared under controlled conditions, and fed a mixed ration based on grass

silage, hay, straw, soybean and corn, with the objective of a weight gain of 1000 g/d. The animals were weighed, blood sampled and submitted to a pericaudal biopsy to get 2 g fat samples two weeks after their arrival in the experimental facility (day 0, sampling A) and bimonthly during the decontamination period (59, 125 and 188 days after starting the decontamination; samplings B, C, and D respectively). The fat samples were stored at -20 °C before being analysed.

TEQ values of DL-PCBs + PCDD/Fs in pericaudal fat were measured, in order to monitor and evaluate the efficiency of the decontamination procedure. The analyses were carried out on pericaudal fat because pericaudal biopsies are minimally invasive, and the animals rapidly recover. In addition, a correlation between the dioxins level in caudal and internal (perirenal) fat was demonstrated (EU-RL, 2009), and it was proposed that the easily accessible subcutaneous fat represents a reliable estimate of adipose tissue contamination by pollutants (Kim et al., 2011).

Eight one year old Charolaise heifers reared at the "Domaine experimental de la Bouzule (Meurthe et Moselle, 54)", i.e. far away from any known PCBs source, were also included in the study as control group and sampled on December 2011 (sampling K1) and March 2012 (sampling K2). Charolaise heifers can differ from the Limousine heifers for fat body mass, and for other general characteristics, so they were used as "control" only because represent heifers never exposed to PCB pollution and reared in the experimental farm where the decontamination took place.

Blood samples were centrifuged at 500 g (20 min; 4 °C), and sera were used for measuring the concentration of non-enzymatic antioxidants (Retinol, alpha-Tocopherol, and Ascorbate), and the activities of enzymatic antioxidants, superoxide dismutase (SOD) and glutathione peroxidase (GPx), here used as indices of the antioxidant defence system. The total antioxidant capacity (TAC) was also assessed, as it well reflects the overall antioxidative potential of the whole organism (Kankofer et al., 2010), and effectively describes the equilibrium between pro-oxidants and antioxidants in blood (Ghiselli et al., 2000). Oxidative modifications were monitored by measuring serum concentrations of nitro-tyrosine (N-Tyr) and protein-bound carbonyls (PC), for evaluating the extent of oxidative damage to protein, and level of lipid hydroperoxides (LPOs) for assessing the extent of lipid peroxidation, induced by the interaction of free radicals with polyunsaturated fatty acids. Haptoglobin and TNF-alpha concentrations were measured as inflammation markers.

Protein concentration in each serum sample (previously diluted 1:80 in 130 mM NaCl, 20 mM Tris-HCl, pH 7.4) was measured by Bradford assay (Bradford, 1976).

DL- DL-PCBs and NDL-PCB analysis PCBs and NDL-PCB analysis

Chemical analyses of PCBs were performed at the French National Reference Laboratory (LABERCA, ONIRIS, Nantes) according to the requirements of the quality assurance parameters of the Commission Directive 2002/69/EC and 2002/70/EC of July 2002 laying down the sampling methods

and the methods of analysis for the determination of PCBs in foodstuffs and feeding stuffs, respectively. Moreover, analyses were performed upon an accredited system ISO 17025. All the methods used have been validated and are accredited ISO 17025. Furthermore, this research project was conducted under a certified system ISO 9001 v. 2000 standard. Briefly, fat samples and feed (~2–5 g) were weighed, and 18 ¹³C-labelled PCBs from Cambridge Isotope Laboratories and Wellington Laboratories were added as internal standards to each sample. The solid samples were extracted by pressurized liquid extraction (ASE 300, Dionex, Sunnyvale, CA, USA), using mixed toluene (Picograde — 1350 Promochem) and acetone (Picograde — 1142 Promochem) at 70/30 (v/v). Final measurement of PCBs was performed by GC-HR-MS (gas chromatograph-HP-5 β 90) from Hewlett Packard (Palo Alto, CA, USA). The mass spectrometer (JMS 700 D, Jeol, Tokyo, Japan) was set at a resolution of 10,000, in electron ionization mode.

Single Ion Monitoring (SIM) was used to record the two most abundant signals of the molecular ion (³⁵Cl and ³⁷Cl isotopic contribution). A DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μ m film thickness) from J&W was used in splitless mode.

The GC temperature programme was 120 °C (3 min), 20 °C min⁻¹ to 170 °C (0 min), 3 °C min⁻¹ to 245 °C (0 min) and finally 20 °C min⁻¹ to 275 °C (7 min). The GC programme for Signals was Diok V2 software. All these values were automatically corrected by taking into account the recovery rate of the ¹³C labelled molecules. The PCBs detection limits in the different tissues and serum analysed including all congeners were better than 30 fg g⁻¹ of the matrix.

Determination of antioxidants and lipid hydroperoxides (LPOs)

Ascorbate (Asc) concentration in serum samples was measured by HPLC, using the anion exchange column Nucleosil 100-NH₂, as previously described (Spagnuolo et al., 2011). Retinol (Ret) and alpha-Tocopherol (Toc) levels were titrated by HPLC, using the reverse phase C18 column Nova-PAK C18, according to a published procedure (Spagnuolo et al., 2011). The total antioxidant capacity (TAC) was measured by the Trolox Equivalent Antioxidant Capacity Assay, according to Miller et al. (1993). Samples were reacted with the radical 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) [ABTS], and the antioxidant capacity was measured as change of the absorbance at 734 nm, and expressed as EIM concentration of Trolox equivalents (Spagnuolo et al., 2001).

LPO concentration was measured by a colorimetric quantitative assay, using the Lipid Hydroperoxide Assay Kit of Cayman Chemical, according to the manufacturer's instructions

Determination of nitro-tyrosine (N-Tyr)

Nitrated protein levels in serum samples were measured by ELISA, essentially according to a published procedure

(Cigliano et al., 2014). Briefly, samples were diluted (1:500, 1:1500, 1:3000, and 1:9000) with coating buffer (7 mM Na₂CO₃, 17 mM NaHCO₃, 1.5 mM NaN₃, pH 9.6), and incubated in the wells of a microtitre plate overnight at 4 °C. Standard curves were obtained with serial dilutions of nitrated BSA. N-Tyr was detected by incubation with Rabbit anti-N-Tyr antibody (1:1500 dilution in 130 mM NaCl, 20 mM Tris-HCl, 0.05% Tween 20, pH 7.3, supplemented with 0.25% BSA; 1 h, 37 °C), followed by GAR-HRP (1:3000 dilution). Colour development was monitored at 492 nm. Data were reported as nmol of N-Tyr per mg of proteins.

Determination of protein-bound carbonyls

PC concentration in serum was titrated by ELISA. Protein derivatization, sample dilution, and immunodetection with Rabbit anti-DNP antibody were carried out according to Cigliano et al. (2014). Data were reported as nmol of carbonyls per mg of proteins.

Evaluation of glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities

GPx activity was measured indirectly by a coupled reaction with glutathione reductase (GR), using the glutathione peroxidase assay kit of Cayman Chemical, according to the manufacturer's instructions. GPx activity was expressed as nmol of NADPH oxidized per minute per ml of sample. SOD activity was measured with the superoxide dismutase assay kit of Cayman Chemical, according to the manufacturer's instructions. SOD activity was expressed Unit/ml. One unit of SOD is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical.

Haptoglobin (Hpt) and TNF-alpha titration

Hpt concentration in individual serum samples was measured by ELISA (Spagnuolo et al., 2014). Samples were diluted

(1:4000, 1:8000; 1:16,000) with coating buffer, and incubated in the wells of a microtitre plate (overnight, 4 °C). Hpt was detected by Rabbit anti-Hpt IgG (1:1500 dilution; 1 h, 37 °C), followed by GAR-HRP (1:4500 dilution, 1 h, 37 °C). The calibration curve was obtained by assaying the immunoreactivity of 6, 3, 1.5, 1, 0.75, 0.5, 0.25 ng of commercial Hpt standard.

TNF-alpha concentration was titrated by sandwich ELISA. Aliquots (50 Eil; 12 Eig/ml) of mouse anti-bovine TNF-alpha IgG were coated into the wells of a microtitre plate (2 h, room temperature). The plate was extensively washed and blocked (1 h, 37 °C) by incubation with 1% BSA in PBS (140 mM NaCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 2.7 mM KCl). Then aliquots (50 Eil) of serum samples (dilution 1:10, 1:30 in PBS) were incubated into the wells (overnight, 4 °C). TNF-alpha was detected by incubation with biotinylated mouse anti-TNF-alpha IgG (1:100 dilution; 2 h, 37 °C) followed by treatment with Streptavidin-HRP (1:1500 dilution; 1 h, 37 °C). The calibration curve was obtained by assaying the immunoreactivity of 20, 10, 5, 2.5, 1, 0.5 pg of recombinant bovine TNF-alpha.

Statistical analysis

The time effect linked to the PCB levels in fat was analysed using the MIXED procedure of SAS, the individual animal being the experimental unit. The student t test was used to compare the mean values between time points.

The samples for measurement of SOD and GPx activities, PC, N-Tyr, Hpt, TNF-alpha, or LPO concentration were processed in triplicate. The titration of Toc, Ret, and Asc was carried out on duplicates. Values were expressed as mean ± SEM. Significance of statistical differences was evaluated by one-way ANOVA, followed by Dunnett's test, for comparing data from samplings performed at days 59, 125 and 188 to data from sampling performed at day 0, or by Tukey's test for multiple comparisons, using the GraphPad Prism 5.01 programme (Graph Pad Software, San Diego, CA, USA). Differences were considered statistically significant when the two-sided P value was less than 0.05. The GraphPad Prism 5.01 programme was also used to calculate correlation coefficient (Pearson's r).

1. Results

Animal growing performances and contamination profile in pericaudal fat

During the 6-month experimental period, the average live weight of the animals increased from 337 to 524 kg, i.e. a mean daily weight of about 1000 g/d. This weight gain was in line with expectations for one year old Limousine heifers.

The analysis of PCDD, PCDF, DL-PCBs, and NDL-PCBs was carried out on pericaudal fat samples essentially because the European Union Regulation indicated Maximum Residue Levels of PCB legally admitted in bovine fat. In addition, the amount of PCBs stored in adipose tissue occurs as a result of bioaccumulation of these toxins over a lifetime of exposure, and it was suggested that the measurement of fat-adjusted values accurately describes the total burden of PCBs residing in adipose tissue throughout the body (Patterson et al., 1988).

As shown in Table 1, both DL-PCBs and NDL-PCBs significantly contributed to bovine contamination. In particular, at the start of the experimental period (time 0, sampling A; two weeks after the removal of the animals from the contaminated area and their arrival to the uncontaminated experimental farm) TEQ value of DL-PCBs and PCDD/Fs was 25.34 ± 1.405 pg/g fat (Table 1). This value was about 6 fold higher than that legally permitted, fixed at 4 pg/g fat (European Union Regulations No 1881/2006, 1259/2011). DL-PCBs provided by far the highest contribution to the TEQ value (about 96%; Table 2), while PCDD/Fs represented less than 4% of the total TEQ (0.945 ± 0.063 pg/g of fat). The high proportion of DL-PCBs was probably due to the origin of the contamination (ingestion of forages contaminated by the emissions of an industrial plant specialized in treatment and decontamination of PCB).

The analysis of contamination profile (Table 3) demonstrated that the more abundant congener among DL-PCBs was PCB 126 (92% of the DL-PCBs), followed by PCB 169 (5.5%) and

PCB 118 (1.6%). In addition, the more represented congeners among PCDD/Fs were 2,3,4,7,8-PeCDF (33%), and 1,2,3,7,8-PeCDD (25%), followed by 2,3,7,8-TCDD (9.6%), 1,2,3,6,7,8-HxCDD (9.2%) and 1,2,3,4,7,8-HxCDF (8.5%).

Depletion curve of DL-PCB + PCDD/Fs (Fig. 1) is described by the equation $y = 20.522e^{-0.011x}$, and demonstrates that half life of these compounds is 44 days. Further, 148 days were necessary to reach the threshold value for DL-PCB + PCDD/Fs (4 pg WHO-TEQ/g fat), and at the end of the experimental period, all animals were considered decontaminated, as TEQ value in pericaudal fat was lower than that legally admitted (European Union Regulation No 1259/2011). Depletion curves of more abundant congeners among PCDD/Fs are shown in Supplementary Fig. S1. Depletion curves of more abundant congeners among DL-PCBs are shown in Supplementary Fig. S2. Half lives of PCB 126, PCB 169 and PCB 118 were calculated and they were found to be 43, 40, and 59 days respectively.

NDL-PCB concentration at the beginning of the decontamination period (165.9 ± 14.42 ng/g of fat) was about 4 fold higher than that legally admitted (Table 1), thus strongly contributing to bovine contamination. As shown in Table 4, the highest contribution to NDL-PCB contamination was from PCB 153 (42%), PCB 138 (29%), and PCB 180 (28%). Depletion curve of NDL-PCB (Fig. 2), described by the equation $y = 132.87e^{-0.01x}$, demonstrates that half life of these compounds is 47 days, and the threshold value of NDL-PCBs (40 ng/g of fat) was reached within 120 days. The specific contamination profile (Table 4) demonstrated that the more abundant congeners were PCB 153 (42.4% of total; calculated half life 47 days), followed by PCB 138 (29.3%; calculated half life 49 days) and PCB 180 (27.9%; calculated half life 46 days). Depletion curves of each congener are shown in Supplementary Fig. S3.

Analysis of serum antioxidants and total antioxidant capacity

Serum concentrations of Ret, Toc, and Asc, the total antioxidant capacity (TAC), as well as GPx and SOD activities were measured as markers of the antioxidant defence system (Table 5).

Ret concentration was positively correlated with Toc concentration only in control animals ($r = 0.805$, $P = 0.016$, sampling K1; $r = 0.930$, $P = 0.007$, sampling K2), and in the samplings performed 125 (sampling C) and 188 (sampling D) days after starting the decontamination process (C , $r = 0.752$, $P = 0.027$; D , $r = 0.769$, $P = 0.035$). As depicted in Table 5, the concentrations of Toc, Ret, and Asc were significantly lower in samples collected at the start of experimental period (day 0; sampling A) with respect to either the control (Toc, $25.88 \pm 0.47\%$; Ret, $47.40 \pm 0.21\%$; Asc, $35.50 \pm 3.51\%$; $P = 0.001$) or to animals sampled during the decontamination period (Toc, $18.54 \pm 0.78\%$; Ret, $47.82 \pm 3.07\%$; Asc, $30.51 \pm 1.27\%$; $P = 0.001$). No statistically significant differences between values measured in samples from control cows and those collected from animals undergoing decontamination (sampling B, C, or D) were recorded.

Similarly the TAC, and the activities of SOD and GPx were significantly more elevated ($55.36 \pm 3.69\%$, $53.05 \pm 0.87\%$,

and $83.48 \pm 5.69\%$ respectively; $P = 0.001$) in serum from control cows than in samples taken at the beginning of decontamination (A), and no differences between control samplings and samplings taken during decontamination (B, C and D) were revealed.

A positive correlation between TAC and Asc concentration was found in control cows and in animals undergoing decontamination (K1, $r = 0.853$, $P = 0.007$; K2, $r = 0.868$, $P = 0.025$; B sampling, $r = 0.793$, $P = 0.019$; C sampling, $r = 0.787$, $P = 0.021$; D, $r = 0.912$, $P = 0.004$; data not shown), but not in cows sampled at time 0 (sampling A). Likewise, Asc concentration was positively correlated with SOD activity in unexposed cows and in cows fed the decontamination diet (K1, $r = 0.818$, $P = 0.013$; K2, $r = 0.823$, $P = 0.040$; B, $r = 0.732$, $P = 0.039$; C, $r = 0.876$, $P = 0.004$; D, $r = 0.852$, $P = 0.015$; data not shown). Finally TAC was negatively correlated with total TEQ of DL-PCBs + PCDD/Fs ($r = -0.8523$, $P = 0.007$; Fig. 3, panel A), and with NDL-PCBs ($r = -0.789$, $P = 0.035$; Supplementary Fig. S4, panel A) in samples collected at the beginning of experimental period.

Analysis of serum PC, N-Tyr and LP

As reported in Table 6, serum levels of N-Tyr and PC were found significantly higher in sampling performed at time 0 (A) than in those from control animals ($40.75 \pm 0.38\%$ and $44.78 \pm 1.38\%$ respectively; $P = 0.001$), or in those taken during the decontamination period ($33.35 \pm 2.73\%$ and $43.80 \pm 1.98\%$ respectively; $P = 0.001$). In addition, the level of N-Tyr was higher ($17.43 \pm 0.53\%$; $P < 0.05$) in samples collected 59 days after the start of decontamination process (sampling B) than in samplings from unexposed animals, and it was also higher ($11.66 \pm 1.36\%$; $P < 0.05$) with respect to the samplings performed after 125 (sampling C) and 188 days (sampling D) of decontamination. Conversely, the level of PC did not differ among these groups. When compared with serum samples collected at time 0 (sampling A), statistically significant lower LPO concentrations were detected in the control cows ($51.11 \pm 0.35\%$; $P = 0.001$), as well as in cows under decontamination ($44.09 \pm 1.25\%$; $P = 0.001$). No differences between control samplings and samplings taken during decontamination (B, C and D) were observed.

Serum titre of N-Tyr was negatively correlated with SOD activity, Asc concentration, and TAC in both samplings from control cows, namely K1 (SOD, $r = -0.790$, $P = 0.020$; Asc, $r = -0.866$, $P = 0.006$; TAC, $r = -0.718$, $P = 0.045$), and K2 (SOD, $r = -0.885$, $P = 0.019$; Asc, $r = -0.906$, $P = 0.013$; TAC, $r = -0.898$, $P = 0.015$). Further, the concentration of N-Tyr was negatively correlated with TAC and SOD activity both in contaminated heifers (sampling A, $r = -0.736$, $P = 0.037$; $r = -0.765$, $P = 0.027$, respectively) and in sera collected during the decontamination i.e. B ($r = -0.0718$, $P = 0.041$; $r = -0.853$, $P = 0.007$, respectively), C ($r = -0.899$, $P = 0.002$; $r = -0.784$, $P = 0.042$, respectively), and D ($r = -0.950$, $P = 0.001$; $r = 0.756$, $P = 0.043$).

Finally, both PC and N-Tyr concentrations were correlated with total TEQ of DL-PCBs + PCDD/Fs (sampling A, $r = 0.942$, $P = 0.0005$; $r = 0.880$, $P = 0.004$ respectively; Fig. 3, panels B and C, respectively) and with NDL-PCBs ($r = 0.806$, $P = 0.029$; $r = 0.842$, $P = 0.018$ respectively; Supplementary Fig. S4, panels

B and C, respectively) in samples obtained at the start of decontamination.

Analysis of Hpt and TNF-alpha

As shown in Fig. 4, serum Hpt concentration was found lower in samples collected at the start of decontamination (A) compared with samplings from control cows ($111.9 \pm 1.66\%$; $P < 0.001$), and with samplings taken during the decontamination phase ($80.88 \pm 7.45\%$; $P < 0.01$). No statistically significant differences among control animals and animals undergoing decontamination were found.

TNF-alpha concentration was significantly higher in samples collected at time 0 (A) and after 59 days of decontamination (B) than in those from control animals (A, $46.06 \pm 3.44\%$, $P < 0.001$; B, $35.30 \pm 4.13\%$, $P < 0.05$), or from samplings taken after 125 (C) and 188 (D) days of decontamination (A, $41.46 \pm$

Discussion

This study was carried out on a group of Limousine heifers that were accidentally exposed to environmental pollutants by the ingestion of forages contaminated by the emissions of an industrial plant, specialized in treatment and decontamination of equipments, transformers, and oils contaminated by PCBs. The pollution was identified as "PCBs contamination" by a control performed by the local prefecture. The heifers were removed from the contaminated area and housed in an experimental facility located far away this area for a decontamination attempt.

In this context it is worth mentioning that DL-PCBs and NDL-PCBs exert numerous and different toxic effects. DL-PCBs act essentially via binding to AhR (Alsharif et al., 1994; Mandal, 2005), that leads to activation of CYP1A and CYP1B genes (Safe, 1986; Whitlock, 1990; Machala et al., 1998; Matsumura, 2003; Guruge et al., 2009). NDL-PCBs have little or no affinity to the AhR, bind to or activate the constitutive androstane receptor and/or the pregnane X receptor, leading to induction of other CYP enzymes (Waxman, 1999; Masahiko and Honkakoski, 2000; Kliewer, 2003), and display a different toxicological profile (Westerink, 2014). Despite the differences in toxicological profile and mechanisms of action, both DL-PCBs and NDL-PCBs are known to induce oxidative stress and inflammatory response (Dutta et al., 2012; Wens et al., 2011; Ferrante et al., 2015; Westerink, 2014; Abella et al., 2015).

Our goal was to evaluate the changes in specific markers of blood redox homeostasis and inflammation during the decontamination process. To this aim, we carried out a longitudinal investigation by analysing serum and fat samples collected during a six month period (days 0, 59, 125 and 188). Serum samples were used for assessing oxidative and inflammatory status, fat samples for defining the extent of contamination at each time point. We also characterized redox and inflammatory status of eight Charolaise heifers reared in the experimental farm where the decontamination took place. Although Charolaise differ from Limousine heifers for general characteristics, we regarded them as "control" group only because they never ingested contaminated forages.

At the start of experimental period (day 0), TEQ values of DL-PCBs + PCDD/PCDF and NDL-PCB amount in pericaudal fat

3.08% , $P < 0.001$; B, $29.77 \pm 3.69\%$, $P < 0.05$) (Fig. 4). Values from control cows and from samplings C and D did not significantly differ. Further, Hpt concentration was correlated with total TEQ of DL-PCBs + PCDD/Fs (Fig. 5) and with NDL-PCBs (Supplementary Fig. S5) in both sampling A (DL-PCBs + PCDD/Fs, $r = -0.735$, $P = 0.038$, Fig. 5, panel A; NDL-PCBs, $r = -0.913$, $P = 0.004$, Supplementary Fig. S5, panel A), and in sampling B (DL-PCBs + PCDD/Fs, $r = -0.946$, $P = 0.0004$, Fig. 5, panel B; NDL-PCBs, $r = -0.763$, $P = 0.028$, Supplementary Fig. S5, panel B). Similarly, TNF-alpha level correlated with total TEQ of DL-PCBs + PCDD/Fs (Fig. 5) and with NDL-PCBs (Supplementary Fig. S6) in both sampling A (DL-PCBs + PCDD/Fs, $r = 0.881$, $P = 0.004$, Fig. 5, panel A; NDL-PCBs, $r = 0.895$, $P = 0.007$, Supplementary Fig. S6, panel A) and in sampling B (DL-PCBs + PCDD/Fs, $r = 0.791$, $P = 0.019$, Fig. 5, panel B; NDL-PCBs, $r = 0.756$, $P = 0.03$, Supplementary Fig. S6, panel B).

were 6 and 4 fold higher, respectively, than those legally admitted, so bovine were classified as "exposed" to PCBs. DL-PCBs represented about 96% of the total TEQ, and PCB 126 was the more abundant congener detected, with an estimated half life of 43 days. The highest contribution to NDL-PCB contamination was found to be from PCB 153, PCB 138, and PCB 180, whose half lives were 47, 49 and 56 days respectively. The initial value of both DL- and NDL-PCB progressively decreased during the examined period (3 fold at day 59, 5 fold at day 125, 8 fold at day 188), thus indicating that the procedure of decontamination was effective. Threshold values of DL-PCB + PCDD/Fs and NDL-PCBs were reached within 148 days and 120 days respectively, therefore at the end of the experimental period (188 days) bovine were considered decontaminated. In the experimental period a significant weight gain occurred (184.0 ± 9.82 kg, $P < 0.0001$), thus suggesting that decontamination depended on both a dilution process and the excretion of the pollutants from the animal organism.

The blood redox status of the cows was characterized by evaluating specific indices of the antioxidant defence system and oxidative damage to lipids and proteins. In line with previously published data (Hassoun et al., 2002; Slezak et al., 2000; Lai et al., 2010; Spagnuolo et al., 2012), serum concentrations of Ret, Toc and Asc, as well as the TAC and the activities of both SOD and GPx were found significantly lower (about 47%, 26%, 36%, 55%, 53% and 83%) in samples collected at day 0 from PCB contaminated heifers than in control heifers. The levels of antioxidants significantly increased (about 30%) as decontamination goes on, and were found similar to those of unexposed bovine just after 59 days.

Ret and Toc are dietary liposoluble antioxidants, and their serum concentrations were expected to be correlated (Olmedilla et al., 1997; Cigliano et al., 2014). Actually, a correlation was found only in serum samples collected in the last phase of the experimental period (days 125 and 188), when both DL- and NDL-PCB concentrations in fat were within the legal limits, and namely 5–8 fold lower than those measured at time 0. Interestingly, Asc concentration was positively correlated with both TAC and SOD activity in samples collected during decontamination, irrespective of the sampling time (day 59, 125, or 188), but not in the samples obtained at time 0. Taken together, these findings suggest that

a severe perturbation of the blood redox homeostasis occurred before starting the decontamination, and that the restoration of physiological concentrations of liposoluble antioxidants might occur more slowly than the other antioxidants here analysed. As expected, a greater extent of oxidative modifications to protein and lipid fractions was detected at the beginning of the experimental period than during decontamination. In particular, PC and LPO concentrations were remarkably higher at day 0 with respect to any other sampling of the decontamination period and of the control cows. As LPO are produced by the oxidative attack of free radicals on polyunsaturated fatty acids, and PC may be generated by reactions of proteins with aldehydes originated during lipid peroxidation processes (Uchida and Stadtman, 1993), our results suggest that lipid peroxidation and its intermediates might play a crucial role in determining oxidative modifications during acute exposure to environmental pollutants, here assessed by the concentrations of DL- and NDL-PCBs. Interestingly, the levels of N-Tyr, which is considered the footprint of protein oxidative damage induced by peroxynitrite, correlated well with the concentrations of DL- and NDL-PCBs. Indeed, serum N-Tyr concentration was just reduced of about 28% after 59 days of decontamination procedure. Further, N-Tyr concentration in samples obtained at day 125 and 188, was significantly lower not only than that at day 0, but also than that at day 59, when TEQ and NDL-PCB concentrations were still over the legal limit (Table 1). Therefore, N-Tyr could represent a useful index for monitoring the decontamination procedure. Finally, TAC, PC and N-Tyr levels correlated with fat content of both DL- and NDL-PCBs only at the start of decontamination.

So we suggested that such parameters could contribute to identify animals exposed to these pollutants, to evaluate the extent of animal exposure, and to monitor the decontamination process.

TNF- α is a proinflammatory multifunctional cytokine released in response to several stress signals (Chen and Goeddel, 2002; Wajant et al., 2003). TNF- α plays an important role in acute dioxin-induced toxicity, and, although the cross-talk between TNF- α and dioxin signalling cascades is a complex, cell-type specific regulated process (Haarmann-Stemmann et al., 2009), studies in mice, sheep, and guinea pigs demonstrated that the exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin was associated with the increase of serum TNF- α levels (Gasiewicz and Neal, 1979; Clark et al., 1991; Moos et al., 1997). In addition, it was recently reported that NDL-PCBs induce TNF- α production (Ferrante et al., 2015). Accordingly, and similar to the trend observed for N-Tyr, higher levels of serum TNF- α were detected in samplings performed at days 0 (about 44%) and 59 (about 33%) as compared to those obtained at days 125 and 188, suggesting that the observed variations of this cytokine might be associated with changes of DL- and NDL-PCBs. In line with this hypothesis, TNF- α level correlated with DL- and NDL-PCB amounts measured at time 0 and after 59 days of decontamination.

Hpt is an acute phase protein with innate antioxidant and immunomodulatory activity, and also acts as a molecular chaperone, inhibiting the inappropriate self-association of proteins induced by oxidation or heat (Saeed et al., 2007;

Quaye, 2008). Hpt concentration in plasma was reported to be down-regulated in humans exposed to polycyclic aromatic hydrocarbons or to 2,3,7,8-tetrachlorodibenzo-p-dioxins (Kim et al., 2004). Our investigation revealed that Hpt concentration was significantly lower in serum samples collected at time 0 than in those collected during the decontamination. More to the point, serum Hpt was negatively correlated with fat levels of DL- and NDL-PCBs measured at day 0 and 59, thus supporting the hypothesis that the extent of down-regulation of this protein depends on the degree of exposure to pollutants, as assessed by PCBs concentration in pericaudal fat. As Hpt stably binds free haemoglobin, thus preventing haemoglobin-related oxidative damage, and also limits the release of haem, which exacerbates oxidative stress (Quaye, 2008), the lower Hpt concentration measured at time 0, when bovines are considered "exposed" to pollutants, might further contribute to the reduction of effectiveness of the antioxidant body defences.

Although the group of Charolaise represents a control only because never exposed to pollution as the Limousine heifers here examined, the finding of significant differences between the two groups, at time 0, led us to suppose that exposure to PCBs, confirmed by the measurements in fat samples, might induce remarkable physiological changes. As oxidative stress contributes to health disorders, and affects zootechnical and/or reproductive performance of dairy cows (Lomba, 1996; Bernabucci et al., 2002, 2005; Castillo et al., 2005; Wilde, 2006; Wathes et al., 2012), the perturbation of oxidative status might have negative implications for animal health and reproduction, and might compromise animal welfare. Interestingly, our results demonstrate that a decontamination procedure, based on the feeding of a controlled diet, is effective within 148 days, and is able to restore the blood homeostasis.

It is worth to underline that the blood parameters analysed in our study may be affected by oxidative stress induced by different kinds of pollutants, such as sulphur oxides, nitrogen oxides, hydrocarbons, carbon monoxide, and transition metals. However, we propose that, in the limits of a contamination characterized by measuring DL- and NDL-PCBs, serum TAC, N-Tyr, PC, Hpt and TNF- α levels could represent useful tools for identifying exposed animals, because their levels correlate with PCB concentration at the starting point of investigation. We report here, for the first time, that the serum concentrations of two inflammatory markers, Hpt and TNF- α , correlate with TEQ value and NDL-PCB amount not only at time 0, but also during decontamination, until PCB concentrations are higher than those legally permitted (day 59). So we propose that Hpt and TNF- α , in our experimental conditions, could be considered reliable markers of animal exposure. Finally, we propose that N-Tyr and TNF- α , whose serum concentrations still differ from those of control animals until fat TEQ and NDL-PCBs are over the legal limit, could be used as biological monitoring markers of the decontamination procedure.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2015.10.104>.

Table 1. PCDD/F + DL-PCB and NDL-PCB levels in pericaudal fat of Limousine heifers under decontamination.

	A	B	C	D
TEQs (PCDD/Fs + DL-PCBs; pg/g of fat)	25.34 ± 1.41***	8.33 ± 0.35.	4.87 ± 0.22	3.13 ± 0.18
NDL-PCBs (ng/g of fat)	165.9 ± 14.42**	56.86 ± 2.88	33.78 ± 1.50	24.16 ± 1.42
Days from starting decontamination	0	59	125	188
Date of sampling (mm, dd, yy)	09/15/11	11/18/11	01/23/12	03/27/12
N	8	8	8	8

Eight one year old Limousine heifers exposed to organic pollutants were removed from the contaminated area and housed in an experimental farm far away from any PCBs source. Analysis

were carried out at the start of the experimental period (day 0; A), and then bimonthly through a six months period (day 59,125, 188; B, C, D).

TEQs: toxic equivalents; PCDDs: polychlorinated dibenzo-para-dioxins; DL-PCBs: dioxin-like polychlorinated biphenyls; PCDFs: polychlorinated dibenzo-furans; NDL-PCBs: non-dioxin-like polychlorinated biphenyls.

*** P < 0.001 vs B, C, or D.

** P < 0.001 vs B, C, or D.

Table 2. Profile of fat contamination by PCDDs, PCDFs and DL-PCBs.

	A	B	C	D
Total of PCDDs	0.43 ± 0.033	0.26 ± 0.017	0.21 ± 0.030	0.16 ± 0.021
Total of PCDFs	0.50 ± 0.040	0.35 ± 0.029	0.23 ± 0.017	0.17 ± 0.013
OMS TEQ DL-PCBs	24.3 ± 1.364	7.71 ± 0.323	4.42 ± 0.199	2.76 ± 0.176
Total TEQs (PCDDs/F + PCB DL)	25.3 ± 1.405	8.33 ± 0.353	4.86 ± 0.215	3.10 ± 0.184
Days from starting decontamination	0	59	125	188
N	8	8	8	8

Eight one year old Limousine heifers exposed to organic pollutants were removed from the contaminated area and housed in an experimental farm far away from any PCBs source. Analysis were carried out at the start of the experimental period (day 0; A), and then bimonthly through a six months period (day 59, 125, 188; B, C, D). TEQs: toxic equivalents; PCDDs: polychlorinated dibenzo-para-dioxins; DL-PCBs: dioxin-like polychlorinated biphenyls; PCDFs: polychlorinated dibenzo-furans. Data are expressed as pg per g of pericaudal fat.

Table 3. Congeners analysis.

	A	B	C	D
<i>PCDD</i>				
2,3,7,8-TCDD	0.091 ± 0.005	0.057 ± 0.009	0.054 ± 0.006	0.051 ± 0.010
1,2,3,7,8-PeCDD	0.232 ± 0.030	0.147 ± 0.012	0.100 ± 0.027	0.070 ± 0.010
1,2,3,4,7,8-HxCDD	0.016 ± 0.001	0.010 ± 0.001	0.013 ± 0.002	0.005 ± 0.0010
1,2,3,6,7,8-HxCDD	0.087 ± 0.010	0.036 ± 0.003	0.029 ± 0.002	0.024 ± 0.003
1,2,3,7,8,9-HxCDD	0.014 ± 0.001	0.009 ± 0.001	0.009 ± 0.0005	0.006 ± 0.0005
HpCDD	0.011 ± 0.001	0.007 ± 0.0008	0.005 ± 0.0004	0.012 ± 0.003
OCDD	0.0008 ± 0.0001	0.0007 ± 0.00008	0.0006 ± 0.00004	0.0004 ±
Total of PCDDs	0.438 ± 0.033	0.265 ± 0.017	0.210 ± 0.030	0.166 ± 0.021
Days from starting decontamination	0	59	125	188
<i>PCDF</i>				
2,3,7,8-TCDF	0.024 ± 0.006	0.012 ± 0.004	0.005 ± 0.0007	0.022 ± 0.003
1,2,3,7,8-PeCDF	0.003 ± 0.0007	0.007 ± 0.0009	0.003 ± 0.0003	0.002 ± 0.0004
2,3,4,7,8-PeCDF	0.313 ± 0.019	0.156 ± 0.017	0.106 ± 0.010	0.085 ± 0.005
1,2,3,4,7,8-HxCDF	0.080 ± 0.011	0.092 ± 0.011	0.058 ± 0.004	0.027 ± 0.004
1,2,3,6,7,8-HxCDF	0.039 ± 0.005	0.041 ± 0.004	0.024 ± 0.001	0.017 ± 0.002
1,2,3,7,8,9-HxCDF	0.005 ± 0.0008	0.004 ± 0.0006	0.004 ± 0.0004	0.004 ± 0.0004
2,3,4,6,7,8-HxCDF	0.027 ± 0.002	0.030 ± 0.003	0.019 ± 0.002	0.015 ± 0.001
1,2,3,4,6,7,8-HpCDF	0.015 ± 0.003	0.009 ± 0.001	0.009 ± 0.0006	0.004 ± 0.001
1,2,3,4,7,8,9-HpCDF	0.002 ± 0.0003	0.002 ± 0.0002	0.002 ± 0.0003	0.0008 ± 0.0001
OCDF	0.0005 ± 0.00009	0.0004 ± 0.00006	0.0003 ± 0.00003	0.0002 ±
Total of PCDFs	0.508 ± 0.040	0.353 ± 0.029	0.231 ± 0.017	0.172 ± 0.013
OMS TEQ PCDD/F	0.945 ± 0.063	0.619 ± 0.046	0.441 ± 0.040	0.339 ± 0.032
Days from starting decontamination	0	59	125	188
<i>DL-PCB</i>				
PCB77	0.0009 ± 0.0001	0.0007 ± 0.00009	0.0004 ± 0.00004	0.0004 ±
PCB81	0.0007 ± 0.00009	0.0002 ± 0.00003	0.0001 ± 0.000009	0.0001 ±
PCB126	22.39 ± 1.236	7.033 ± 0.296	4.027 ± 0.188	2.468 ± 0.163
PCB169	1.330 ± 0.129	0.430 ± 0.026	0.249 ± 0.010	0.186 ± 0.012
Total of coplanares	23.73 ± 1.327	7.465 ± 0.315	4.276 ± 0.196	2.654 ± 0.171
PCB105	0.037 ± 0.003	0.011 ± 0.0009	0.006 ± 0.0005	0.005 ± 0.0004
PCB114	0.006 ± 0.0006	0.003 ± 0.0001	0.002 ± 0.00006	0.001 ± 0.00008
PCB118	0.398 ± 0.028	0.160 ± 0.006	0.099 ± 0.004	0.066 ± 0.004
PCB123	0.003 ± 0.0001	0.0012 ± 0.00007	0.0007 ± 0.00004	0.0007 ±
PCB156	0.122 ± 0.013	0.040 ± 0.002	0.024 ± 0.001	0.019 ± 0.001
PCB157	0.018 ± 0.002	0.007 ± 0.0004	0.004 ± 0.0002	0.005 ± 0.0004
PCB167	0.062 ± 0.005	0.021 ± 0.001	0.013 ± 0.0004	0.009 ± 0.0006
PCB189	0.0189 ± 0.002	0.006 ± 0.0005	0.003 ± 0.0001	0.002 ± 0.0002
Total of non-coplanares	0.665 ± 0.050	0.249 ± 0.010	0.152 ± 0.006	0.109 ± 0.006
OMS TEQ DL-PCB	24.39 ± 1.364	7.714 ± 0.323	4.428 ± 0.199	2.763 ± 0.176
Total TEQ (PCDD/F + PCB DL)	25.34 ± 1.405	8.333 ± 0.353	4.869 ± 0.215	3.102 ± 0.184
Days from starting decontamination	0	59	125	188

Eight one year old Limousine heifers exposed to organic pollutants were removed from the contaminated area and housed in an experimental farm far away from any PCB source.

Analysis were carried out at the start of the experimental period (day 0; A), and then bimonthly through a six months period (days 59, 125, 188; B, C, D).

TEQs: toxic equivalents; PCDDs: polychlorinated dibenzo-para-dioxins; DL-PCBs: dioxin-like polychlorinated biphenyls; PCDFs: polychlorinated dibenzo-furans.

Data are expressed as pg per g of pericaudal fat.

The more abundant congeners are marked in bold.

Table 4. Congeners analysis.

	A	B	C	D
PCB28	0.276 ± 0.030	0.20 ± 0.035	0.104 ± 0.010	0.061 ± 0.010
PCB52	0.231 ± 0.039	0.21 ± 0.039	0.088 ± 0.011	0.118 ± 0.019
PCB101	0.388 ± 0.044	0.31 ± 0.057	0.143 ± 0.020	0.203 ± 0.032
PCB138	48.57 ± 4.136	16.8 ± 1.012	10.12 ± 0.451	6.738 ± 0.446
PCB153	70.26 ± 5.742	23.9 ± 1.176	14.59 ± 0.668	10.43 ± 0.567
PCB180	46.29 ± 5.167	15.4 ± 0.818	8.733 ± 0.441	6.614 ± 0.456
Total of NDL-PCBs	165.9 ± 14.42	56.8 ± 2.884	33.78 ± 1.502	24.16 ± 1.422
Days from starting decontamination	0	59	125	188
N	8	8	8	8

Eight one year old Limousine heifers exposed to organic pollutants were removed from the contaminated area and housed in an experimental farm far away from any PCBs source. Analysis were carried out at the start of the experimental period (day 0; A), and then bimonthly through a six months period (days 59, 125, 188; B, C, D). The more abundant congeners are marked in bold.

Table 5. Markers of the antioxidant defence system in serum of heifers

	A	B	C	D	K1	K2
Ret (µg/ml)	0.48 ± 0.02 **	0.72 ± 0.02	0.73 ± 0.01	0.68 ± 0.01	0.71 ± 0.01 ***	0.71 ± 0.08 ***
Toc (µg/ml)	1.48 ± 0.04 **	1.73 ± 0.02	1.76 ± 0.03	1.77 ± 0.03	1.85 ± 0.03 ***	1.87 ± 0.03 ***
Asc (µM)	6.06 ± 0.11 **	7.76 ± 0.20	7.95 ± 0.16	8.01 ± 0.12	8.00 ± 0.11 ***	8.42 ± 0.27 ***
GPx (nmol/min/ml)	95.73 ± 4.08 **	164.1 ± 6.7	169.2 ± 8.5	167.0 ± 8.3	170.2 ± 15 ***	181.1 ± 14 ***
SOD (U/ml)	1.10 ± 0.04 **	1.55 ± 0.06	1.57 ± 0.04	1.62 ± 0.04	1.67 ± 0.06 ***	1.69 ± 0.09 ***
TAC (µM)	92.24 ± 2.76 **	133.0 ± 3.5	139.4 ± 4.2	141.0 ± 3.8	139.9 ± 3.2 ***	146.7 ± 2.6 ***
Days from starting decontamination	0	59	125	188		
Date of sampling (mm, dd, yy)	09/15/11	11/18/11	01/23/12	03/27/12	12/15/11	03/29/12
N	8	8	8	7	8	6

Ret, Retinol; Toc, α-Tocopherol; Asc, Ascorbate; TAC, total antioxidant capacity; GPx, glutathione peroxidase activity; SOD, superoxide dismutase.

Eight one year old Limousine heifers exposed to organic pollutants were removed from the contaminated area and housed in an experimental farm far away from any PCBs source.

Samplings were carried out at the start of the experimental period (day 0; A), and then bimonthly through a six months period (days 59, 125, 188; B, C, D).

K1 and K2 are samplings, taken at different times, from Charolaise heifers reared in the same experimental farm far away from any PCBs source, and regarded as controls.

*** Avs K1 or K2, P < 0.001.

** Avs B, C, or D, P < 0.001

Table 6. Markers of oxidative stress in serum of heifers

	A	B	C	D	K1	K2
PC (nmol/mgP)	12.39 ± 0.17**	7.29 ± 0.32	7.11 ± 0.15	6.48 ± 0.44	6.67 ± 0.22***	7.01 ± 0.31***
N-Tyr (nmol/mgP)	8.82 ± 0.09**	6.33 ± 0.19	5.50 ± 0.21	5.80 ± 0.36	5.26 ± 0.18***	5.19 ± 0.19***
LPO (µM)	5.83 ± 0.16**	3.16 ± 0.21	3.21 ± 0.24	3.40 ± 0.29	2.87 ± 0.18***	2.83 ± 0.24***
Days from starting decontamination	0	59	125	188		
Date of sampling (mm, dd, yy)	09/15/11	11/18/11	01/23/12	03/27/12	12/15/11	03/29/12
N	8	8	8	7	8	6

Eight one year old Limousine heifers exposed to organic pollutants were removed from the contaminated area and housed in an experimental farm far away from any PCBs source.

Samplings were carried out at the start of the experimental period (day 0; A), and then bimonthly through a six months period (days 59, 125, 188; B, C, D).

K1 and K2 are samplings, taken at different times, from Charolaise heifers reared in the same experimental farm far away from any PCB source, and regarded as controls.

PC, protein-bound carbonyls (nmol per mg of protein); N-Tyr, nitro-tyrosine (nmol per mg of protein); LPO, lipid hydroperoxides (µM).

*** Avs K1 or K2, P < 0.001.

** Avs B, C, or D, P < 0.001.

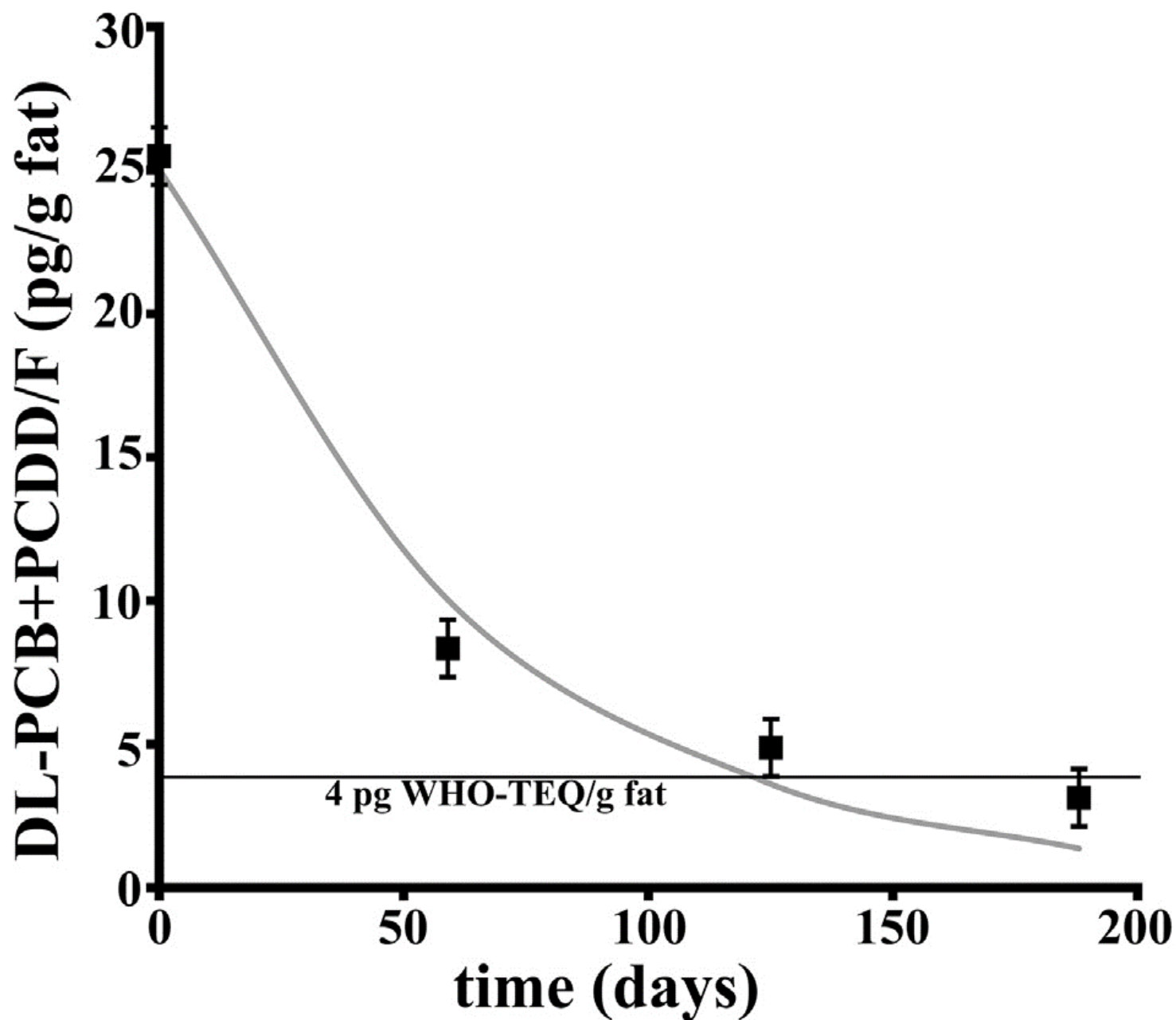


Figure 1. Decontamination kinetic of DL-PCB and PCDD/F in growing Limousine heifers.

Eight one year old Limousine heifers were removed from a contaminated farm, located close to an industrial plant, and housed in an experimental facility located far away this area, for six months, for a decontamination attempt. The extent of individual contamination was evaluated by measuring total toxic equivalent (TEQ) values of DL-PCBs + PCDD/F s in pericaudal fat of each animal at the start of the decontamination process (day 0), and bimonthly during the decontamination period (days 59, 125 and 188). Data are reported as mean \pm standard deviation. The threshold limit for DL-PCB + PCDD/F (4 pg WHO-TEQ/g fat; European Union Regulation No 1259/2011) is indicated by a continuous line.

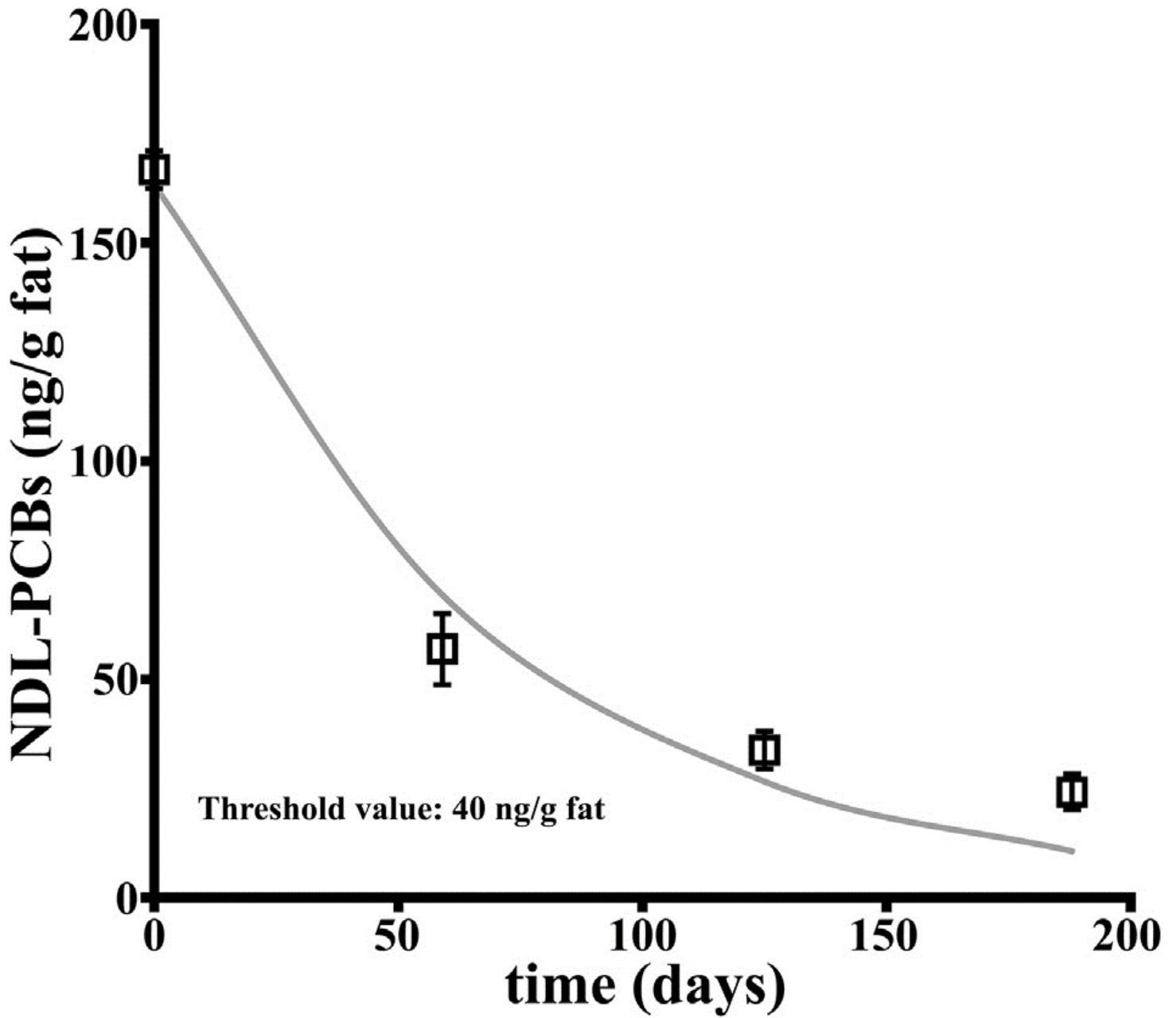


Figure 2. Decontamination kinetic of NDL-PCBs in growing Limousine heifers

Eight one year old Limousine heifers were removed from a contaminated farm, located close to an industrial plant, and housed in an experimental facility far away from this area, for six months, for a decontamination attempt. The extent of individual contamination was evaluated by measuring NDL-PCBs amount in pericaudal fat of each animal at the start of the decontamination process (day 0), and bimonthly during the decontamination period (days 59, 125, and 188). Data are reported as mean \pm standard deviation. The threshold limit for NDL-PCBs (40 ngWHO-TEQ/g fat; European Union Regulation No 1259/2011) is indicated.

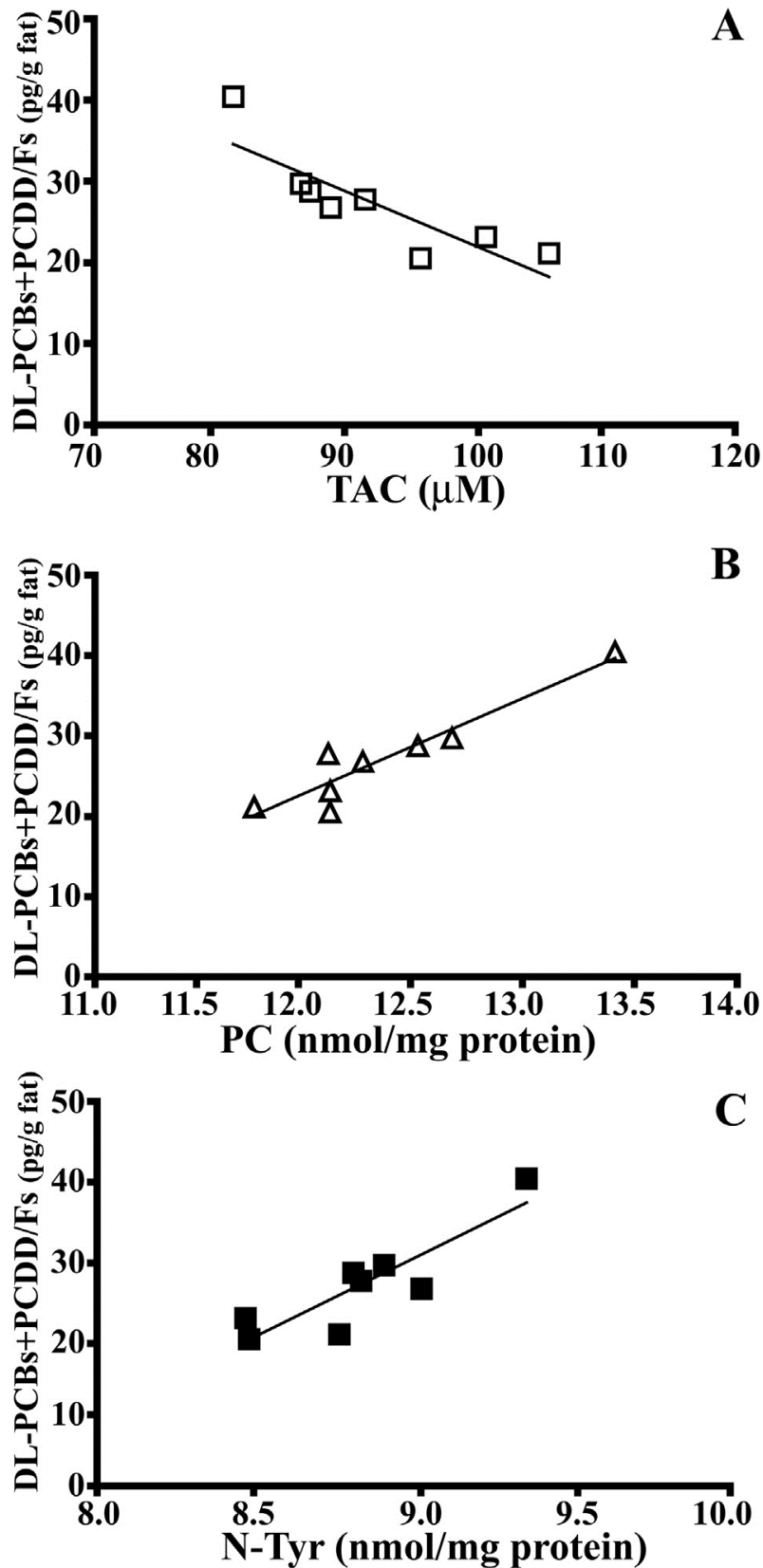


Figure 3. Correlation between total TEQ in pericaudal fat and specific serum markers of redoxhomeostasis.

Panel A. The total antioxidant capacity (TAC) was measured in serum samples from Limousine heifers at the starting point of decontamination (day 0), and expressed as μM concentration of Trolox equivalents. Each sample was analysed in triplicate, and the average value was calculated. Deviations over 5% from the mean were not found. Total toxic equivalent (TEQ) values of DL-PCBs + PCDD/F in pericaudal fat of each animal was measured and reported as pg/g fat . The statistical programme Graph Pad Prism 5.01 performed Pearson correlation analysis and the calculation of P ($r = -0.8523$, $P = 0.007$). Panel B. The level of PC was measured, by ELISA, in serum samples collected at the starting point of decontamination (day 0), and expressed as $\text{nmol/mg of protein}$. The statistical programme Graph Pad Prism 5.01 performed Pearson correlation analysis and the calculation of P ($r = 0.942$, $P = 0.0005$). Panel C. The concentration of N-Tyr was measured, by ELISA, in serum samples collected at the starting point of decontamination (day 0), and expressed as $\text{nmol/mg of protein}$. The statistical programme Graph Pad Prism 5.01 performed Pearson correlation analysis and the calculation of P ($r = 0.880$, $P = 0.004$).

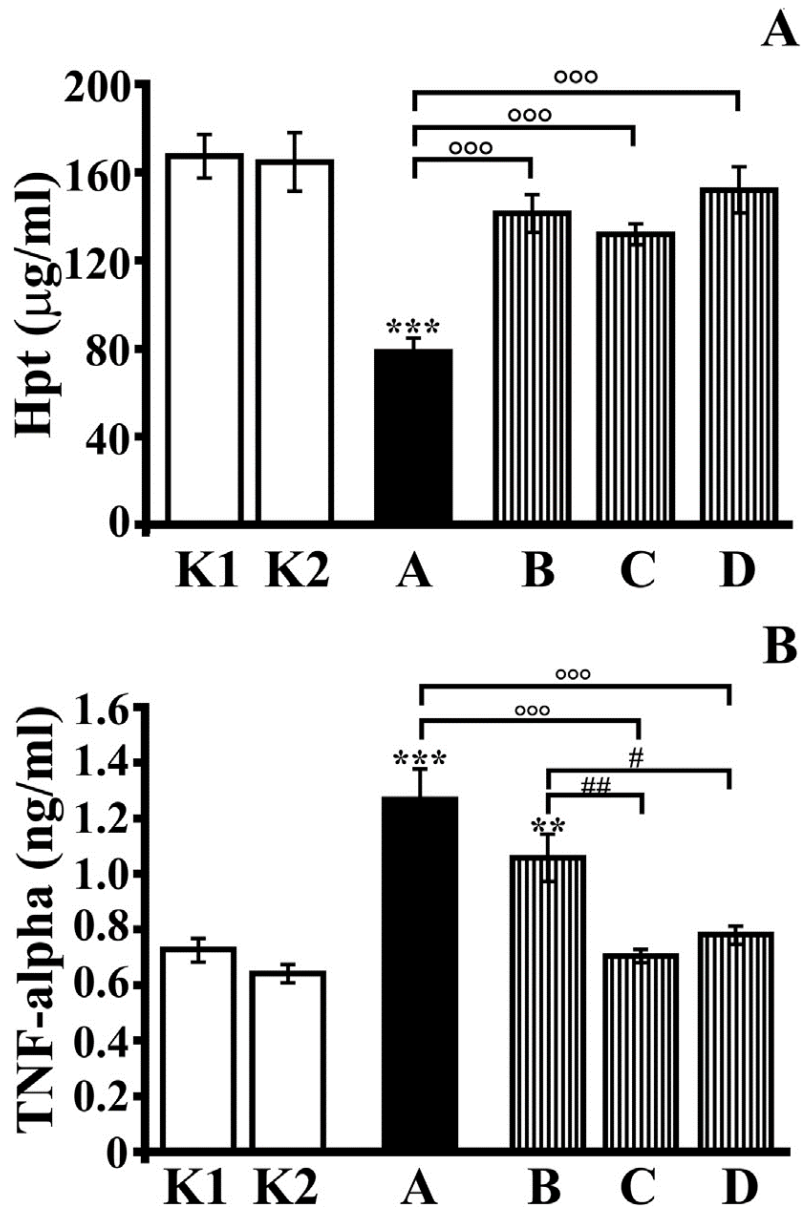


Figure 4. Serum concentration of Hpt and TNF-alpha. Hpt (panel A) and TNF-alpha (panel B) concentration in serum was measured by ELISA.

K1 and K2 (open bar), samplings from Charolaise heifers reared in the experimental farm far away from any PCBs source, here regarded as controls. A (fullbar), sampling from Limousine heifers exposed to organic pollutants collected at the start of the experimental period (day 0). B, C and D (bar with vertical lines), samplings performed after 59 (B), 125 (C), and 188 (D) days of decontamination. ***P < 0.001 vs A (from Dunnett's test). *P < 0.05 vs B; ##P < 0.01 vs B (from Tukey's test). **P < 0.01 vs K1 and K2; ***P < 0.001 vs K1 and K2 (from Tukey's test).

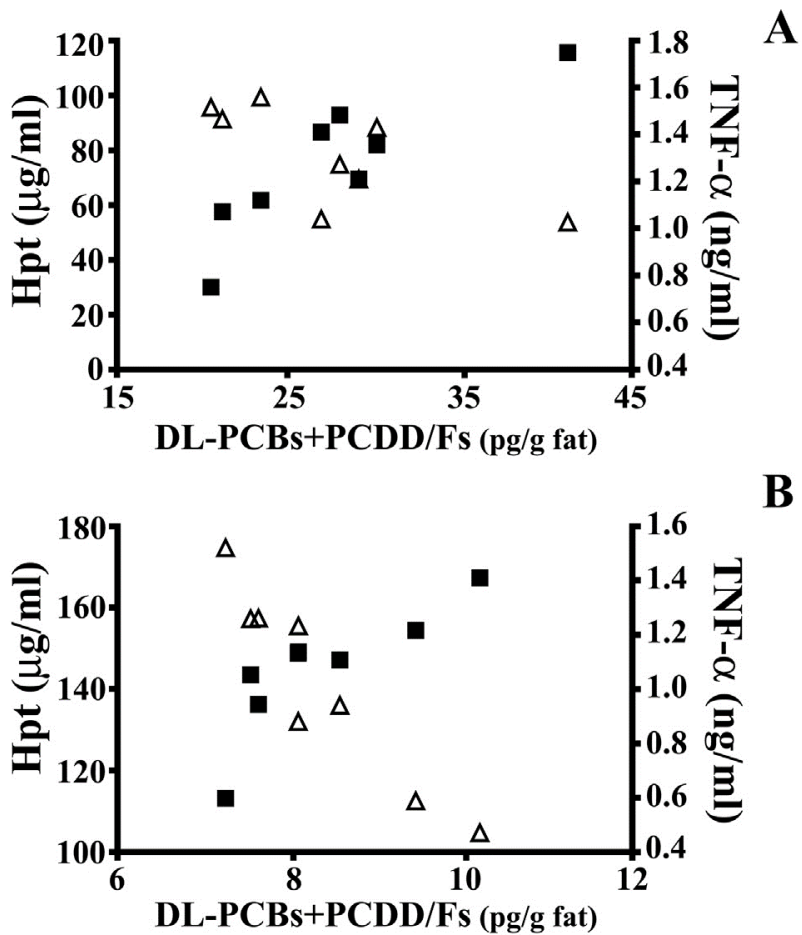


Figure 5. Correlation between total TEQ in pericaudal fat and serum concentration of Hpt or TNF-alpha. Panel A. Hpt (open triangles) and TNF-alpha (full squares) concentrations were measured, by ELISA, in serum samples from heifers at the start of decontamination (day 0). Each sample was analysed in triplicate, and the average value was calculated. Deviation over 5% from the mean were not found. Total toxic equivalent (TEQ) values of DL-PCBs + PCDD/F in pericaudal fat of each animal are reported as pg/g fat. The statistical programme Graph Pad Prism 5.01 performed Pearson correlation analysis and the calculation of P (Hpt, $r = -0.735$, $P = 0.038$; TNF-alpha, $r = 0.881$, $P = 0.004$ respectively). Panel B. Hpt (open triangles) and TNF-alpha (full squares) concentrations were measured, by ELISA, in serum samples collected 59 days after starting decontamination. The statistical programme Graph Pad Prism 5.01 performed Pearson correlation analysis and the calculation of P (Hpt, $r = -0.946$, $P = 0.0004$; TNF-alpha, $r = 0.791$, $P = 0.019$).

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3. Acknowledgements

The study was supported by CISIA-VARIGEAV project (Caratterizzazione e valorizzazione delle risorse genetiche animali e vegetali della Campania e della Sardegna finalizzate allo sviluppo della filiera bufalina mediante approcci multidisciplinari ed

innovativi; grant number 191/2009), and by POR FESR 2007/2013 project (DEDICA: Sviluppo di tecniche biomolecolari di screening per la messa in evidenza dell'esposizione a diossine e sostanze diossino-simili in vacche da latte).