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Aroclor 1254 inhibits the chemiluminescence response of peritoneal cavity cells from sharpsnout sea bream (*Diplodus puntazzo*)

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ABSTRACT

Chronic exposure to polychlorinated biphenyls (PCBs) affect the immune system of fish and could lead to a decreased disease resistance. The effects of Aroclor 1254, PCB mixtures, on the *Diplodus puntazzo* innate immunity were examined by assaying the zymosan stimulated chemiluminescence response (CL) of peritoneal cavity cells (PCCs) at various times (1, 24, 48 h and 1–4 weeks) from intraperitoneal injection of the xenobiotic (1 mg kg^{-1} body weight). Controls were performed by assaying cells from medium-treated fish. Since the kinetic of the chemiluminescence response showed the highest peak at 25 min after the zymosan stimulation of the cells, the values found at that time were considered. The CL enhancement observed at 1 h after the treatment with xenobiotic was followed by a decreased response at 24 h and appeared to be lower at 1–4 weeks when compared to the CL response of the control, suggesting a protracted effect of PCBs on the peritoneal cavity. Since PCCs incubated *in vitro* for 1 h with 0.05 and 0.1 μg ml^{-1} Aroclor showed an enhanced CL, the effect of the xenobiotic could be exerted on the cell responsiveness to zymosan. It is known that fish CL response of PCCs can be imputed to phagocyte (macrophages and neutrophils) activation, these cells and their responsiveness to zymosan can be used in immunotoxicology assay to monitor the fish health in polluted environment.

1. Introduction

Aroclor 1254 is one of several congeners present in the mixture of commercial polychlorinated biphenyls (PCBs) manufactured for several industrial purposes until banned in the late 1970s [1]. Nevertheless it has been estimated that approximately 10% of the PCBs ever produced are still environmentally available [2]. The high environmental persistence of this product, its ability to be bio-accumulated and its bio-magnification in the food chain renders it a biological risk [3,4].

Although the cessation of PCB production, congeners remain widely distributed in aquatic systems where they are released by slow desorption from sediments causing harmful biological effects on aquatic species being absorbed through epidermis, gills and contaminated food [5,6]. Despite they are ubiquitous aquatic contaminants, some toxicological effects of PCBs have been underestimated [7] and little information is available regarding the impact of PCBs on fish innate immune system [8]. On the other hand, it has been shown that exposure to PCB congeners depress both humoral and cell mediated immune responses in mammalian host [9–11].

Phagocytosis is a main barrier of the innate immune system to contrast pathogens, and phagocyte sensitivity to xenobiotics can affect fish healthy.

Phagocytic cells are widely distributed in tissues of fish immune system [12–14], and they show morphological characters of mammalian neutrophils and macrophages [15,16] including an enhanced respiratory burst after activation [16]. Following a challenge, phagocytes increase their oxygen consumption and reactive oxygen intermediates (ROIs) can be measured using the chemiluminescence (CL) assay.

In brief, a specific luminescence is amplified when luminol permeates cells and reacts with a variety of ROIs including superoxide, hydroxyl peroxide, singlet oxygen, etc. [17,18]. Xenobiotics can affect these cells and phagocyte respiratory burst appears to be especially sensitive to their effect [19].

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Fish peritoneal cavity cells (PCCs) have been characterized morphologically [20] as macrophages, neutrophils, eosinophils and lymphocytes. In sea bass, the peritoneal neutrophils and macrophages are phagocytes [21], and in rainbow trout they are activated following injection of inflammatory agents [22,23]. PPCs can be recruited by intraperitoneal injection of an isosmotic medium, stimulated *in vitro* with zymosan, and their respiratory burst response evaluated by the CL assay [21].

In the present paper we report the effect of intraperitoneal exposure to a non-lethal concentration of Aroclor 1254 injected into peritoneal cavity of sharpsnout sea bass *Diplodus puntazzo*. At first, the kinetic of CL response of PPCs after zymosan challenge was displayed, then, the CL response as an effect of the xenobiotic was evaluated after *in vivo* or *in vitro* treatments. The PCB doses we used had no effect upon fish survival as well as on cell vitality up to the end of the experiments.

2. Materials and methods

2.1. Fish

Fish weighing 250–300 g were obtained from a fish farm (Ecoittica, TP, Italy), maintained with re-circulating water (0.4 L min^{-1}) at 17 ± 2 °C. Fishes, in the department aquaria, were randomly separated into four fibre–glass tanks (1000 L, 21 fish each) and acclimated at constant environmental conditions for at least 2 weeks, and fed with commercial pellets (Trouvit, Hendrix SpA). Feeding, lighting, water temperature and acclimation to housing were controlled. All experiments were performed in full compliance with the national rules and guidelines (D. Lgs 116/92 and subsequent amendments) and international European Commission Recommendation guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC).

2.2. PCB in vivo exposures

A stock Aroclor 1254 (PCB, Ultra Scientific) solution was prepared in dimetilsulfoxide (1 mg in 1 ml DMSO) and diluted 1:4 with Hanks balanced salt solution (HBSS: NaCl 190 mM, KCl 5.36 mM, glucose 5.54 mM, KH_2PO_4 0.44 mM, Na_2HPO_4 0.56 mM; pH 7.6, 370 mOsm kg^{-1}). After anaesthesia with 0.05% 3-aminobenzoic acid ethyl ester methanesulfonate (MS222, Sigma–Aldrich, Germany) in sea water, the ventral surface of fish were disinfected with 70% ethyl alcohol and a volume (1 ml) of xenobiotic solution (HBSS-DMSO-PCB) containing 250 μg PCB (1 mg kg^{-1} body weight) were injected into the peritoneal cavity of fish (IP: intraperitoneal injection) of tank 1.

Fish from the tank 2 were injected with a same volume of HBSS-DMSO, and the fish from tank 3 were injected with HBSS. Peritoneal cavity cells were collected after 1, 24, 48 h and 1, 2, 3 e 4 weeks. For each time, three distinct experiments were carried out and 189 fish in total were examined.

Fish ($n = 3$) from tank 4 were not treated and were used for *in vitro* experiments.

2.3. Preparation of peritoneal cavity cells (PCCs)

After each fish treatment the PCCs were harvested according [21], 10 ml of isotonic (370 mOsm kg^{-1}) Leibovitz medium (Leibovitz L15 medium containing 2% foetal calf serum, 100 units penicillin ml^{-1}, 100 units streptomycin ml^{-1} and 10 units heparin ml^{-1}) were injected into the peritoneal cavity. After massaging the ventral surface for 10 min, the medium containing the PCCs was withdrawn with a syringe. The cells were collected by centrifuging at 400 *g* for 10 min at 4 °C, counted in a haemocytometer and evaluated for cell mortality by Trypan blue exclusion test (0.01% in Leibovitz medium). The cell number was standardised to 2.5×10^5 cells ml^{-1} in HBSS and PCCs from treated and untreated fish assayed for their CL response.

Differential counts of PPCs from three fish for each treatment were carried. Cell smears were performed on glass slide, stained with May Grunwald-Giemsa solution, and 100 microscopical fields (2.6×10^3 cells in total) were examined under a light microscope DLMB Leica, (Wetzlar, D). Data were reported as percent ± SD.

2.4. ROI measurement by a chemiluminescence assay (CL)

The response of a constant number of PPCs (2.5×10^5 cells ml^{-1} in HBSS) was measured as ROI production using a luminol-amplified CL assay. Luminol solution was prepared as a potassium salt by adding 0.014 g luminol, 0.78 g potassium hydroxide and 0.618 g boric acid to 10 ml of distilled water and bringing to 100 ml by the addition of HBSS. This stock solution was stored in the dark and diluted 1:100 in HBSS to give a working luminol solution. Zymosan (β-glucan of yeast cell wall, Sigma) was boiled for 30 min in phosphate buffered saline (PBS) and then centrifuged for 5 min at 400× *g*, suspended in 50 ml of PBS and stored until use with a maximum storage time of 2 weeks [18]. The final concentration of zymosan was 1 mg ml^{-1}.

Aliquots of 0.5 ml PCC suspension (1.125×10^5 cells in HBSS) were mixed with 0.5 ml of the luminol solution in propylene vials. Cell response was displayed after adding 0.5 ml of zymosan. Controls were performed as samples of the reaction mixture in which, in turn, luminol, zymosan or cells were substituted by medium.

A scintillation counter (Beckman LS 1800) was used to measure the CL. Data are expressed as counts per minute (cpm).

2.5. PCB in vitro exposures

A PCC suspension (2.5×10^5 cells ml^{-1}) was exposed according to Hammond [24]. From three untreated fish was incubated (v/v) with 0.05 or 0.1 μg ml^{-1} Aroclor in H-D, for 1 h at 18 °C, and then assayed for their CL response. Cells incubated with HBSS or H-D were the controls. The experiments were performed in triplicate.

2.6. Statistical analysis

Data, *in vivo*, obtained from each fish group were presented as mean ± SD of three distinct experiments in which were used 3 fish for each experiment, and statistically analysed by the Student's *t*-test. Differences were considered statistically significant for $P < 0.05$. Data, *in vitro*, are the mean of three distinct experiments in which were used 3 fish for each experiment.

3. Results

3.1. Effect of IP injection of Aroclor 1254 on fish survival

The treatment with HBSS, H-D or H-D-PCB had no effect on behaviour, feeding and fish survival up to 4 weeks post injection and the cell mortality, evaluated by the Trypan blue exclusion test, was unchanged (<1%).

3.2. Kinetic of CL response of PCCs from fish inoculated with HBSS or PCB

PCCs were withdrawn from fish treated for 1 h and were incubated with zymosan and CL measured at 5 min interval up to 60 min (Fig. 1). the PCCs from HBSS treated fish showed a CL values

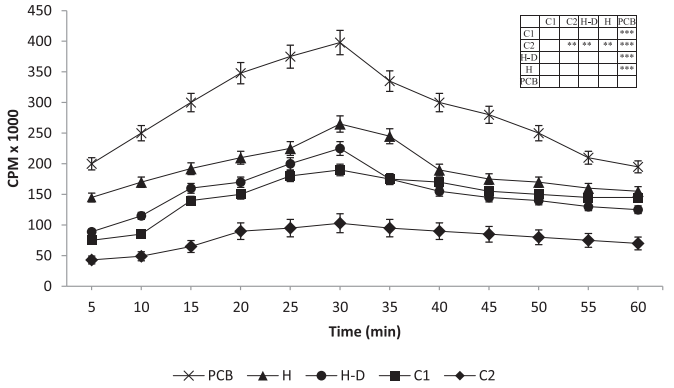

Fig. 1. Chemiluminescence response of PCCs at the same cellular number (2.5×10^5 ml^{-1}) activated by zymosan after intraperitoneal injection of HBSS −▲− (H), H-D −●− (H-D), H-D-PCB −×− (PCB), for 1 h. Control = PCCs from untreated animals with−■− (C1), and without−◆− (C2) zymosan. In table are shown the significance values between the various tests; ** = $p < 0.01$; *** = $p < 0.001$.

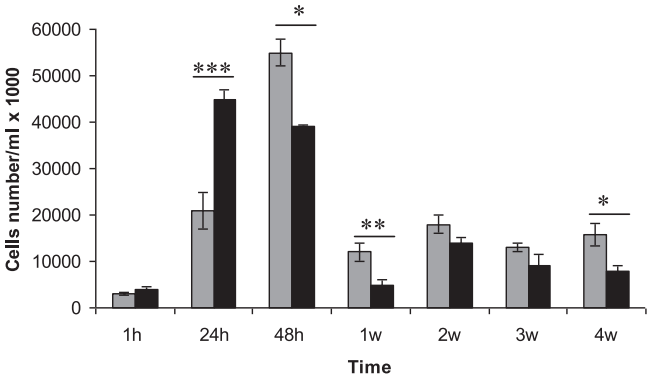

Fig. 3. Number of cells from peritoneal cavity after treatment with HBSS + DMSO (▩1) or HBSS + DMSO-PCB (■) at different times (1, 24, 48 h, 1, 2, 3 and 4 weeks). * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

peaked (220 ± 20 cpm) at 30 min incubation time, then decreased and reached the initial level at 30 min, whereas in the absence of zymosan the cells did not display any significant CL increase until 60 min.

The cells harvested from fish treated with H-D injection, showed a kinetic of CL response similar to the above reported profile and peaked at 30 min reaching a value of 195 ± 18 cpm (Fig. 1). The PCB effect was shown by the significantly ($P < 0.001$) higher CL response compared to that due to HBSS or H-D injection (Fig. 1). As shown in Fig. 1, PPCs collected from fish at 1 h treated fish with H-D-PCB yielded a typical profile but were more sensitive to the zymosan activation and their CL response resulted further amplified. The CL at 30 min was doubled (400 ± 80 cpm) compared to that of PCCs from HBSS fish. The treatment with HBSS containing DMSO did not cause any significant variation in the CL response, and it was at a level similar to that displayed by HBSS injection.

3.3. Effects of H-D or PCB-HD on CL response at various times post injection

Based on the kinetic of the CL response, the values as follows reported were those found at 30 min. Fig. 2 shows the activity of PCCs (2.5×10^5 cells ml^{-1}) at various times after the H-D-PCB treatment (250 μg/fish) or H-D alone. The effect of H-D treatment enhanced the CL activity of PCCs, and such an enhancement was

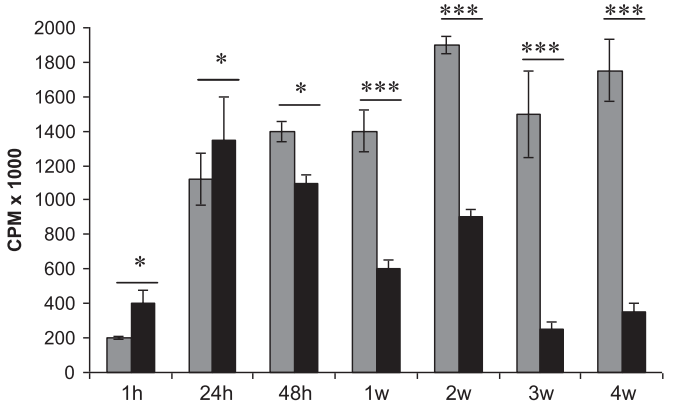

Fig. 2. A costant number (2.5×105 ml^{-1}) of cells from the peritoneal cavity, assayed for CL response after treatment with HBSS + DMSO (▩) and HBSS + DMSO-PCB (■) at different times (1, 24, 48 h, 1, 2, 3 and 4 weeks). * = $p < 0.05$; *** = $p < 0.001$.

protracted until the end of the experiment as shown by the high CL level found at 48 h and at 1–4 weeks (Fig. 2). When the effect of H-D was compared to that of the PCCs from fish treated with H-D-PCB, a CL enhancing effect of PCB was manifest at 1 h and 24 h, then it decreased becoming significantly inhibitory ($P < 0.05$) at 48 h. Such an inhibitory effect was more intense at 1 and 2 weeks ($P < 0.001$) and it was drastic at 3 and 4 weeks when the CL response was at the level found after 1 h.

3.4. PCB, HBSS and HBSS-DMSO exert a recruitment effect on PCCs

To examine the effect of the medium or PCB on cell recruitment into the peritoneal cavity, the PPCs were collected at various times post treatment and counted. The H-D medium exerted an inflammatory effect as shown by the high cell number in the peritoneal cavity (Fig. 3). At 1 h, the cell number (3×10^6 ml^{-1}) did not vary between H-D and H/D-PCB treatments, whereas an increased amount of cells were recruited at 24 h (21×10^6 ml^{-1}) reaching the highest value ($55 \times 10^6 \pm 3 \times 10^3$ ml^{-1}) at 48 h as an effect of the treatment, then decreased ($12 \times 10^6 \pm 2 \times 10^3$ ml^{-1}) at 1–4 weeks even if higher levels were found compared with the cell counts accomplished at 1 h post injection ($3 \times 10^6 \pm 3 \times 10^2$ ml^{-1}).

After 1 h treatment with H-D-PCB, the cell number did not change significantly ($4 \times 10^6 \pm 4 \times 10^2$ ml^{-1}) compared to the H-D injection, whereas it increased ($P < 0.001$) at 24 h ($45 \times 10^6 \pm 2 \times 10^3$ ml^{-1}). However, at 48 h the xenobiotic was less effective than H-D in recruiting cells ($39 \times 10^6 \pm 5 \times 10^2$ ml^{-1}), and from 1 to 4 weeks the cell number lowered showing values ($5 \times 10^6 \pm 1 \times 10^3$ ml^{-1}) inferior than those observed as an effect of H-D injection.

As reported in Table 1, differential count of PPCs from untreated fish ($n = 3$) showed the following cell type proportions: 27.5% ± 1.3% neutrophils, 12.2% ± 1.7% eosinophils, 36.7% ± 1.9% macrophages, and 23.4% ± 1.5% lymphocytes. The cell recruitment

Table 1
Differential cells count of PCCs from untreated (HBSS, H-D) and PCB treated *D. puntazzo* specimens.

Cell type	Number of cell (%)		
	HBSS	H-D	H-D-PCB
Neutrophils	27.6 ± 1.3	32.8 ± 2.2	17.0 ± 1.1[a]
Eosinophils	12.2 ± 1.7	13.0 ± 1.2	17.8 ± 2.3
Macrophages	36.8 ± 1.9	38.5 ± 1.5	43.7 ± 1.8
Lymphocytes	23.4 ± 1.5	15.7 ± 2.1	21.5 ± 2.7

[a] = $P < 0.001$.

at 24 and 48 h due to H-D injection did not concern the relative proportion of a particular cell type (32.8 ± 2.2% neutrophils, 13.0 ± 1.2% eosinophils, 38.5 ± 1.5% macrophages, 15.7 ± 2.1% lymphocytes) (Table 1), whereas H-D-PCB almost halved ($P < 0.001$) the neutrophil proportion (17.0 ± 1.1%).

3.5. *In vitro* effect of PCBs on peritoneal cavity cells

To ascertain if the PCB affected the cellular pathway leading to the ROI production, 2.5×10^5 PCCs ml^{-1} from untreated fish were assayed *in vitro* in the presence of HBSS, H-D or H-D-PCB (0.05–0.1 μg ml^{-1}) for 1 h and then stimulated with zymosan. The cell mortality after *in vitro* different treatments was inferior than 1%.

Although different levels were found, the CL response to zymosan showed a typical kinetic profile like that displayed by PCCs from inoculated fish and the CL peaked at 25 min (Fig. 4). Likewise the effect exerted by *in vivo* treatment, HBSS and H-D amplified the CL response, but the levels they reached were lower than those showed by PCB-treated cells. When the cells were maintained in the presence of 0.1 μg ml^{-1} PCBs the CL response was significantly higher than that displayed by cells treated with 0.05 μg ml^{-1} PCBs, HBSS or H-D (Fig. 4).

4. Discussion

The polychlorinated biphenyls mixture, Aroclor 1254, is environmentally relevant since the congener composition of this mixture is very similar to that bio-accumulated in contaminated fish in the wild [25–27]. A broad spectrum of biological effects, including immunotoxicity, with specific modes of action has been associated with PCB congener mixtures [4,28–30].

The cells obtained from the peritoneal cavity following injection of a physiological medium, represents an useful model for studying the effects of xenobiotics on phagocytes. Likewise in mouse [31], PPCs can be used in toxicological assay and their phagocytic function is useful to evaluate and monitor the innate immune response of fish exposed to xenobiotic. In accordance with these assumptions, we used the peritoneal cavity cells of the sharpsnout sea bream *D. puntazzo* for examining Aroclor 1254 effect on phagocytes.

In a previous paper, we showed that the injection of an isosmotic medium into the sea bass peritoneal cavity caused a prompt influx of cell populations containing high proportion of neutrophils and macrophages [21]. This proportion was significantly higher than that obtained by examining cells separated from blood or organs of the immune system. Furthermore, we showed that *in vitro* cell challenge with zymosan enhanced the respiratory burst of peritoneal cavity phagocytes (macrophages and neutrophils) measured as chemiluminescence response. Such an enhancement was higher than that observed by assaying cells from the other tissues.

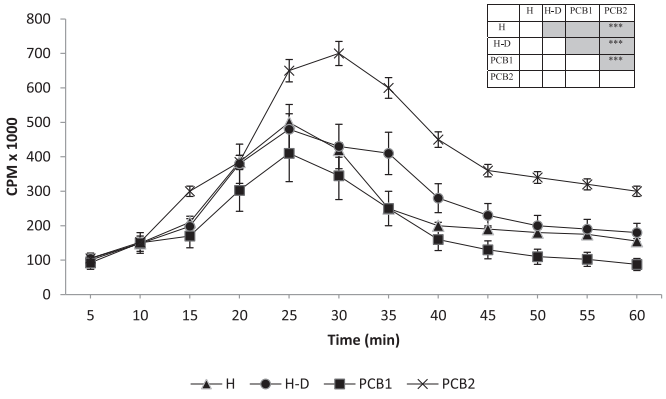

Fig. 4. Peritoneal cavity cells (2.5×10^5 ml^{-1}) stimulated by zymosan after treatment *in vitro* for 1 h with HBSS –◆– (H); HBSS-DMSO –●– (H-D); HBSS-DMSO-PCB 0.05 μg ml^{-1} –■– (PCB1) and H-D-PCB 0.1 μg ml^{-1} –▲– (PCB2). In table are shown the significance values between the various tests; *** = $p < 0.001$.

In this study we show that concentration of PCB, similar to that used for other study [24], are able to modulate the CL response of PPCs. Indeed, the kinetic of CL by PPCs from untreated sharpsnout sea bream (cells collected at 10 min after medium injection) and treated fish (cells collected with the standard method at various time after a PCB intraperitoneal injection) presented a CL profile similar to that reported in sea bass [21]. Just after the cells were incubated with zymosan the CL peaked at 25 min and decreased up to the initial value at 45 min, whereas in the absence of zymosan not significant increase was observed. At 1 and 24 h after the PCB (dissolved in HBSS containing DMSO) was injected, PPCs became more sensitive to zymosan activation and their CL response appeared to be significantly ($P < 0.05$ and $P < 0.05$ respectively) amplified. Since each assay was performed with a standard cell number and the cell mortality did not vary after the treatments, the increased respiratory burst of stimulated cells could be imputed to their enhanced reactivity.

A similar PCB stimulating effect on the phagocytic function was found by Lacroix [32] by assaying American place macrophages.

To ascertain if the CL amplifying effect of PCBs was due to an enhanced cell activity, PCCs from untreated fish were stimulated *in vitro* with zymosan after 1 h incubation with 0.05–0.1 μg ml^{-1} PCB in HBSS-DMSO. This treatment did not cause increased cell mortality. Results showed that the CL profiles (within 60 min) were similar including those obtained by assaying the PPCs from fish challenged *in vivo*. CL values reached the highest levels at 25 min in the presence of PCB, whereas the CL values recorded for both untreated cells and HBSS-DMSO treated cells were significantly lower. When the CL response of PCCs from fish injected with Aroclor 1254 were examined at subsequent times and compared with that of the H-D inoculation, the inhibitory effect of the xenobiotic could be observed at 48 h and at 1–4 weeks.

It is noteworthy that both HBSS and HBSS containing DMSO injected into the peritoneal cavity 24–48 h or 1–4 weeks exerted an inflammatory action. An increased number of PCCs were harvested, mainly at 24 and 48 h, while a constant number of PCCs showed a significant increase in their CL response compared to cells withdrawn from fish just after (10 min) they were injected with HBSS. DMSO has been used as solvent since it shows an exceptionally low toxicity. However, our data show that this substance exerts an evident inflammatory action and it is not free of secondary effect as also reported for its modulating activity on fish oestrogen receptors [33]. The effect of the Aroclor 1254 appeared to be amplificatory of the CL response at 1 h and 24 h, becoming inhibitory at 48 h and at 1–4 weeks when the effect was more evident. Although an increased cell number was found in the peritoneal cavity following PCB injection (mainly at 24 and 48 h), the above reported changes in CL activity observed by assaying a constant cell number suggest that a direct relationship between CL activity and total cell number cannot be drawn. On the other hand, whereas the recruitment due to H-D at 24 h and 1 week did not concern the relative proportion of a particular cell type, PCB injection decreased the neutrophil proportion. Such an effect was mainly observed at 1 week, when the total number of recruited cells was low and the neutrophil proportion was halved compared to H-D controls. Although we were not able to establish the role of decreased neutrophils in CL response of a whole PCC preparation, the possibility exists that the enhanced CL could be due to less numerous neutrophils provided with enhanced responsiveness. In

this respect [21] reported that sea bass PCCs showed a CL response mainly due to neutrophil proportion in the PCC preparations [33].

However, the possibility of an increased activation capacity of macrophages cannot be excluded.

Although we do not know whether PCB treatment directly affected the phagocytic activity of peritoneal phagocytes, the activating or inhibiting effects on the CL response to zymosan suggested an immunosuppressive effect. Results also suggest that the xenobiotic mixture at the used concentrations, could maintain its inhibitory effect on innate immune system during a long time. Alterations in innate immune function upon xenobiotic exposure could result in an impaired immune defence against pathogens or inappropriate inflammatory responses producing increased tissue injury [34]. Finally, the results are in accordance with the exposure to Aroclor mixtures of isolated rat neutrophils which appeared to be activated and undergo oxidative burst through stimulation [31,35].

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