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Effects of polychlorinated biphenyls (PCB) on California sea lion (*Zalophus californianus*) lymphocyte functions upon *in vitro* exposure

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ABSTRACT

Polychorinated biphenyl (PCB) congeners are a cause for concern due to their persistence in the environment, their lipophilic properties that cause them to bio-accumulate in top predators, and their adverse effects on mammalian health. For example, the common urogenital carcinoma reported in California sea lions (*Zalophus californianus*) (CSL) is associated with high tissue levels of PCBs, but the mechanisms responsible for this association are unknown. This study investigated the effect of exposure to six PCB congeners and a congener mix at low and environmentally relevant concentrations on NK cell-like and T cell activity using *in vitro* assays on cryopreserved lymph node mononuclear cells isolated from dead CSL. Non dioxin-like congeners 153 and 180 increased lymphocyte proliferation at 5 and 10 ppm, while congener 138 decreased proliferation by up to 43% at 15 ppm. Dioxin-like PCBs 118 and 169 did not affect lymphocyte proliferation, while the effects of congener 105 depended on the mitogen concentration; these did not correlate with their predicted toxic equivalent factors. NK cell-like activity was affected only by the highest concentration of PCBs tested; it was increased by non-dioxin-like congeners 138 and 153, and decreased by dioxin-like congener 169. The PCB congener mix suggested that the effects of PCB congeners were not simply additive. Our results concur with effects of PCBs reported for other pinniped's lymphocytes and add further experimental support to the observation that dioxin-like PCBs are not the most toxic congeners for marine mammals, contrary to effects in other species. This is the first evidence of *in vitro* suppression of NK cell-like cytotoxicity by a dioxin-like congener in a pinniped. More importantly, the observed results suggest that PCBs can modulate the CSL immune system, increasing exposed individuals' susceptibility to viral and oncogenic challenges.

1. Introduction

California sea lions (CSL, *Zalophus californianus*) have an unusually high prevalence of cancer. In California, urogenital carcinoma is currently detected in approximately 25% of adult stranded CSLs that are subjected to *post-mortem* examination (Deming et al., 2018). The disease has been associated with high blubber levels of polychlorinated biphenyl (ΣPCB) congeners (Ylitalo et al., 2005), and they appear to relate strongly to metastasis (Ylitalo et al., 2005). There is a marked spatial pattern in PCB concentrations in sea lion tissues along the CSL distribution. Levels decrease markedly southwards, being two or three orders of magnitude higher in California, USA (77,000 ng g^{-1} in males and 83,000 ng g^{-1} in females; Ylitalo et al., 2005) than in the Mexican Pacific (2960 ng g^{-1}; Del Toro et al., 2006) and Gulf of California (1600 ng g^{-1} in males, 1300 ng g^{-1} females; Niño-Torres et al., 2009). Interestingly, this pattern coincides with the distribution of observed urogenital carcinoma. Although cellular transformation of the genital epithelium has been reported in CSL from Mexican waters, urogenital carcinoma has not been detected, despite systematic sampling of live animals (Barragán-Vargas et al., 2016). This observation supports the proposed importance of PCB pollutant exposure in cancer development. However, to date, the mechanism(s) through which PCBs contribute to the development of urogenital carcinoma remain unknown.

Organochlorines are known to affect marine mammal health (Brown et al., 2018; Kajiwara et al., 2001) *via* endocrine disruption (Jensen, 2006), reproductive failure (DeLong et al., 1973), and immunosuppression (Desforges et al., 2016; Lahvis et al., 1995). The adverse effects reported for different species include the development of tumors (De Guise et al., 1995). One proposed mechanism for the observed effects is PCB-induced immunomodulation, a phenomenon that has been described in various species (Mori et al., 2006, 2008), and that appears to be concentration-dependent, at least in phocids (Ross et al., 1996a; de Swart et al., 1996). Natural killer (NK) and T cells are particularly important for the detection of transformed cells and prevention of carcinogenesis (Beyer and Schultze, 2006; Ruffell et al., 2010). Thus, if these cells were af-

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fected by exposure to PCBs, cancer could be more likely to develop, as would the probability of infection and dissemination of oncogenic viruses. Taking into account their biological functions, assessing CSL NK cell-like and T lymphocyte activity in response to PCB exposure could help to understand their role in the development of urogenital carcinoma in California sea lions.

The *in vitro* measurement of NK cell-like cytotoxicity has been widely used as an indicator of innate cytotoxicity (Hamerman et al., 2005), antitumorigenic ability, and prognosis of metastasis (Pasero et al., 2015; Takeuchi et al., 2001). In humans, mice and other animal models (Knapp et al., 1993; Mata et al., 2014), NK cell activity is generally measured in circulating blood cells (Bryceson, 2006; Tarazona et al., 2015) using flow cytometry (De Guise et al., 1997; Tarazona et al., 2015) or radioactive Cr^{51} (De Guise et al., 1997). In addition to cytotoxicity assays, lymphocyte proliferation can be measured to assess the adaptive immune system, and has been used for different marine mammal species (*e.g.* De Guise et al., 1998; Lahvis et al., 1995; Levin et al., 2005a; Mori et al., 2006). A combination of two immune assays is currently considered the standard for determining the potential for immune suppression by exposure to a compound (De Guise et al., 2003).

To date, the relatively few studies conducted in cetaceans and pinnipeds have usually been performed on fresh blood samples collected from animals maintained in managed care facilities (De Guise et al., 1997; Ross et al., 1996a) or from free-living individuals captured at sites that are near to the processing laboratory (De Guise et al., 1997). Unfortunately, the logistical difficulties of procuring blood samples from wild animals and processing them immediately after collection, limit the wider application of these cytotoxicity assays for marine mammals, as fresh blood samples will only remain viable for a limited time after collection. Also, when using carcasses, blood is difficult to collect, as it clots quickly after death. However, lymphocytes can also be harvested from lymph nodes from fresh carcasses. Lymph nodes contain high numbers of mononuclear cells (LNMC), including T cells and NK cells (Mason, 1981; Poppema et al., 1981; Fehniger et al., 2003; Freud et al., 2005), and the tissue remains viable for culture up to 24 h after the animal's death (Pugliares et al., 2007). *In vitro* assays using T cells isolated from lymph nodes have been carried out quite frequently (Desforges et al., 2016); however, NK cell activity has not been as widely studied, especially for marine mammals (see Ross et al., 1996b; De Guise et al., 1997).

This study investigated the effect of PCB congener exposure on NK cell and T cell activity using *in vitro* assays on cells harvested from CSL lymph nodes. To test whether environmentally relevant concentrations of PCBs immunomodulate the response of CSL NK cells and T cells *in vitro*, three approaches were used. First, LNMC were examined to see whether they displayed NK cell-like activity against tumor cells. Secondly, NK cell-like activity was examined under different concentrations of individual congeners, as well as a PCB congener mixture. Finally, lymphoproliferation assays were carried out at the same PCB concentrations used for the NK cell-like activity assays.

2. Material and methods

2.1. Collection of samples and isolation of LNMC

Mesenteric lymph nodes were harvested from 13 fresh CSL carcasses and sent to the laboratory at the University of Connecticut to be processed within 24 h of collection. Sampling was conducted under MMPA permit 18786 issued to The Marine Mammal Center (TMMC, Sausalito, CA, USA). All samples were collected from animals undergoing rehabilitation at TMMC. Lymph node samples were stored in complete Dulbecco's Modified Eagle's Medium (see below) and placed on cold packs for shipping. Upon arrival, the lymph nodes were sliced into several sections of $\leq 1\,cm^3$ and cells were extracted by repeated flushes with Hank's Balanced Salt Solution (HBSS; Thermo Fisher Scientific, Grand Island, NY) using a sterile 10 mL syringe and 23 G needle. Erythrocytes were lysed using NH_4Cl (Levin et al., 2007) and mononuclear cells were isolated using Ficoll-Paque 1.077 (GE Healthcare Life Sciences, Pittsburgh, PA) at 990 g for 40 min prior to re-suspending in complete Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) and 10% fetal calf serum (HyClone, GE Healthcare Life Sciences, Pittsburgh, PA). Cells were cryopreserved at 10^7 cells/mL in fetal calf serum with 10% dimethyl sulfoxide (DMSO, Sigma, St Louis, MO), progressively cooled overnight to −80 °C in a CoolCell® cell freezing container (BioCision, San Rafael, CA), and transferred to a liquid nitrogen storage container, where they remained for at least one month prior to the assays being performed.

LNMC from 13 CSL were extracted and cryopreserved. The number of samples needed to achieve a statistical power of 0.80 was calculated considering a beta error of 0.2 and an alpha error of 0.05, yielding 7.85 animals. Thus, the sample size for each treatment was eight, unless a statistical power of 0.80 was achieved using fewer animals.

2.2. Measurement of NK cell-like activity

Although *in vitro* assays have been described to characterize NK cell activity, given the description of NK cell-like cytotoxicity by gamma-delta T cells (Lilienfeld-Toal et al., 2006) and other innate lymphoid cell subsets (Tian et al., 2016), and the use of mixed and uncharacterized cell types from sampled CSL lymph nodes, this study will refer to the description of NK cell-like cytotoxicity. Prior to conducting the assays, the NK cell-like cytotoxicity of CSL LNMC against two tumor cell lines was investigated to determine whether one of the tumor cell lines was more susceptible than the other. LNMCs from two sea lions were quickly thawed in a 37 °C bath and immediately added to 50 mL tubes containing warm complete DMEM. Tubes were centrifuged and washed with DMEM twice, and viability was assessed with Trypan blue and light microscopy. Viability was calculated as [# live cells / (# dead cells + # live cells)] x 100, and was greater than 80% for both sea lions.

Cell lines K-562 (CCL-243™, ATCC, Manassas, VA), and YAC-1 (TIB-160™, ATCC, Manassas, VA) were used as tumor cell targets for LNMC cytotoxicity. K-562 is a human myelogenous leukemia cell line and YAC-1 is a murine lymphoma cell line. Both target cell lines are recognized as generally sensitive to NK cell-like cytotoxicity (Kiessling et al., 1975), and have been used to assess innate cytotoxicity in cetaceans and pinnipeds (De Guise et al., 1997; Levin et al., 2005a; Ross et al., 1996a; Gebhard et al., 2015) and other wildlife species (Rousselet et al., 2013; Desforges et al., 2018). Target cells were cultured in complete DMEM at 37 °C with 5% CO_2. Target cells were passed 1:10 and 1:2 in complete DMEM 48 and 24 h, respectively, prior to each assay.

NK cell-like activity was measured using the mortality of target cells at different LNMC to target cell ratios using flow cytometry as previously described (Desforges et al., 2018). Briefly, 1 mL of target cells (K-562 or YAC-1) were incubated with 10 μL of 3 mM 3,3'-dioctadecyloxabocyanine perchlorate (DiO, Molecular Probes, Grand Island, NY) dissolved in DMSO. Cells were incubated for 20 min at 37 °C and 5% CO_2, washed with DMEM and adjusted to 10^5 cells/mL. The CSL LNMC used as effector cells were adjusted to 10^6 cells/mL, and 1, 0.5, 0.250, 0.125 and 0 mL of this preparation were pipetted into different 5 mL round bottom tubes. The viability of LNMC was above 80% for these samples. The volume of the tubes was completed with DMEM to reach 1 mL and 100 μL of K-562 or YAC-1 cells were added to achieve effector: target (E: T) ratios of 100:1, 50:1, 25:1, 12.5:1. A tube that contained target cells alone was used as a control to assess spontaneous mortality. The E: T mixtures were centrifuged for 30 s at 220 g and then incubated for 150 min at 37 °C and 5% CO_2. All tests were performed in duplicate.

Following centrifugation at 220 g for 10 min at 4 °C, the supernatant was discarded and the cells were re-suspended in 200 μL of phosphate buffer saline solution and placed on wet ice before immediate analysis. A solution of 50 μg/mL of propidium iodide (PI, Molecular Probes, Eugene, OR) was added to each tube immediately prior to acquisition to evaluate mortality of the target cells using two-color (DiO *vs.* PI) flow cytometry. The fluorescence of at least 1000 target cells was read using a FACScan flow cytometer (Becton Dickinson, Rutherford, NJ) and the automated CellQuest software (Becton Dickinson Immunocytometry System, San Jose, CA). Effector cells were identified by

their relative size (forward-scattered light, FSC) and their complexity (side-scattered light, SSC) and distinguished from DiO-labeled target cells, which show higher fluorescence at 530 nm (FL-1). Dead or dying cells incorporate PI due to membrane instability, and they showed higher fluorescence at 630 nm (FL-3). The percent target cell mortality was calculated as: [# dead target cells/(# dead target cells + # live target cells)] x 100. The percent of spontaneous target cell mortality was subtracted from the percent target cell mortality to calculate specific target cell mortality.

Given a previous report that NK cell is enhanced when cryopreserved cells were first rested overnight (Mata et al., 2014), we compared the NK cell-like cytotoxicity of CSL LNMC that were thawed and rested unstimulated overnight at 37 °C to that of cells that were thawed the morning of the cytotoxicity experiment, using YAC-1 cells and an E: T ratio of 50:1.

2.3. Measurement of NK cell-like activity under exposure to PCBs

A 100 μL volume of thawed LNMC (2×10^5) was pipetted into 5 mL round bottom tubes. For each congener, three different biologically relevant concentrations (5 ppm, 10 ppm, 15 ppm in 0.4% DMSO) were tested based on values reported in sea lion blubber (Ylitalo et al., 2005; Del Toro et al., 2006), in addition to an unexposed control of 0.4% DMSO alone. As PCB congeners are commonly found as mixtures in the environment (Robertson and Hansen, 2015), a mixture of congeners was prepared using the relative proportions of the selected pollutants reported for wild CSL (dioxin-like congeners 105, 118 and 169, and non dioxin-like congeners 138, 153 and 180; Table 1). The total concentrations tested for the mixture were 5, 10 and 15 ppm. Individual PCBs or the mixture, as well as complete DMEM, was added to reach a final volume of 200 μL. The tubes were incubated for 3 h at 37 °C with 5% of CO_2. DiO-labeled YAC-1 cells were added at a final E: T ratio of 50:1, after having determined this to be the ideal E: T (see Section 3.1). Cells were incubated for 150 min, centrifuged, and resuspended in fresh DMEM. Target cell fluorescence was acquired on a FACScan flow cytometer as described above (See Section 2.2). All assays were conducted in duplicate.

2.4. Lymphocyte proliferation assays

Concanavalin A (ConA)-induced lymphocyte proliferation was carried out similar to assays previously performed in lymphocytes harvested from pinnipeds and other marine mammals (Mori et al., 2006). For each treatment, cryopreserved LNMCs were thawed, washed twice in complete DMEM and adjusted to 2×10^6. Cells were plated in triplicate in 96 flat bottom wells plates (Falcon, Becton Dickinson, Franklin Lakes, NJ). Two concentrations of ConA, an optimal concentration at 1 μg/mL and a suboptimal concentration at 0.1 μg/mL, were used. The suboptimal mitogen concentrations were selected because they have been shown to be more sensitive at detecting immunotoxicity (Mori et al., 2006). Cells were exposed to the same three concentrations of individual PCB congeners and PCB mixture as described above (see Section 2.3). Cells were incubated at 37 °C with 5% of CO_2 for 48 h before adding BrdU, a thymidine analogue, in order to assess proliferation. ConA and PCB congeners were added simultaneously to the cell culture. Cells were incubated for an additional 18 h and BrdU was detected with a monoclonal antibody and a colorimetric enzymatic reaction (Cell Proliferation ELISA BrdU [colorimetric], Roche Diagnostics, Alameda, CA), as per the manufacturer's instructions. Plates were read with an ELISA plate reader (Multiskan EX v.1.0) at 690 nm with a reference wavelength of 450 nm. The triplicate measurements of the optical densities were averaged and the Stimulation Index was calculated by dividing each result by that of the unstimulated control sample.

Table 1
Relative proportions of each PCB congener in the PCB mix (600 ppm). Concentrations were determined based on Del Toro et al. (2006).

Congener	Concentration in 4.4 mL of complete DMEM	Volume
PCB 105	1.25%	0.0549 mL
PCB 118	3.46%	0.1521 mL
PCB 138	2.21%	0.0972 mL
PCB 153	6.34%	0.2789 mL
PCB 169	0.19%	0.0085 mL
PCB 180	0.19%	0.0085 mL

2.5. Statistical analysis

Shapiro-Wilk tests were used to determine deviations from normality, and equality of variance was assessed by a Brown-Forsythe test (Brown and Forsythe, 1974). Repeated-measure analysis of variance (RM ANOVA) and *post-hoc* Dunnett's tests were used to determine if each PCB concentration was significantly different from the unexposed control. For results that deviated from assumptions of normality, data were normalized by a square root transformation. If, despite transformation, the data did not show evidence of normality and homoscedasticity, data were analyzed using RM ANOVA on Ranks (Gholib et al., 2017). Power was calculated using standard formulas (Chow et al., 2008). All analyses were run on SigmaStat 3.5 (Systat software, Inc.) and graphs were created using GraphPad Prism 7.04 for Windows (GraphPad Software, La Jolla California USA). For all tests, statistical significance was established at $p < 0.05$.

3. Results and discussion

3.1. NK cell-like activity in cryopreserved cells extracted from lymph nodes

A consistent pattern of decreased specific mortality was observed with decreasing E: T ratios for YAC-1 and K-562 cell lines, suggesting that both cell lines were vulnerable after spontaneous mortality was subtracted (Fig. 1). However, YAC-1 cells were approximately three times more sensitive to CSL LNMC cytotoxic activity than K-562 cells ($\mu = 10.12\%$, SD = 4.72 and $\mu = 3.92\%$, SD = 0.26 for 100:1 E: T ratio, respectively; $\mu = 10.47\%$, SD = 4.38 and $\mu = 2.99$, SD = 0.03 for 50:1; $\mu = 7.8{,}4$ SD = 4.87 and $\mu = 3.42$, SD = 0.13 for 25:1; $\mu = 8.23\%$ SD = 4.04 and $\mu = 1.39$ SD = 0.32 for 12.5:1) In all cases, spontaneous mortality remained under 4%. Based on these results, YAC-1 cells were selected to measure NK cell-like activity with PCB exposure (see Section 3.2). The higher reactivity of CSL LNMC to YAC-1 than to K-562 concurs with previous observations in phocids (Ross et al., 1996a; M. Levin, personal communication) and contrasts with reports for cetaceans (De Guise et al., 1997).

We further compared the benefits of resting cells overnight before the cytotoxicity testing. Unlike in humans (Mata et al., 2014), CSL LNMC cytotoxic activity was lower (although not significantly, $\mu = 7.73\%$, SD = 4.44 and $\mu = 5.66\%$, SD = 2.29 for cells not rested, and cells rested overnight, respectively, Fig. 2). Therefore, freshly thawed CSL LNMC were used in further cytotoxic activity assays.

Interestingly, NK cells harvested from human lymph nodes usually lack perforin (Vivier et al., 2008), so they are not expected to exert innate cytotoxicity (Strbo et al., 2003). Based on these observations, it is possible that CSL LNMC synthesize perforin. Whether this is a common finding across marine mammals remains to be seen, and future studies should check cells for cytotoxic activity before using this assay in other species.

3.2. NK cell-like activity with PCBs

NK cell-like activity was not affected significantly by four of the six individual PCB congeners when compared to the control (RM ANOVA; PCB 105, $F_{3,8} = 2.160$, $p = 0.123$, Fig. 3a; PCB 118, $F_{3,8} = 1.618$, $p = 0.215$, Fig. 3b; PCB 153, $F_{3,8} = 2.722$, $p = 0.070$, Fig. 3d, and PCB180, $F_{3,8} = 0.808$, $p = 0.504$; Fig. 3f). PCB 169 decreased NK cell-like activity by 5.2% at 15 ppm (RM ANOVA; $F_{3,8} = 3.327$, $p = 0.039$, Fig. 3e). This reduction in NK cell-like activity is similar to the previous findings in phocids fed with organochlorine polluted herring (Ross et al., 1996b). The biological significance of this effect is unknown, as dioxin-like PCB congeners are underrepresented in free-ranging CSL tissues (Del Toro et al., 2006), however, as stated earlier, these can accumulate quickly in nursing pups (Sørmo et al., 2003). Also, there was a non-significant trend suggestive of

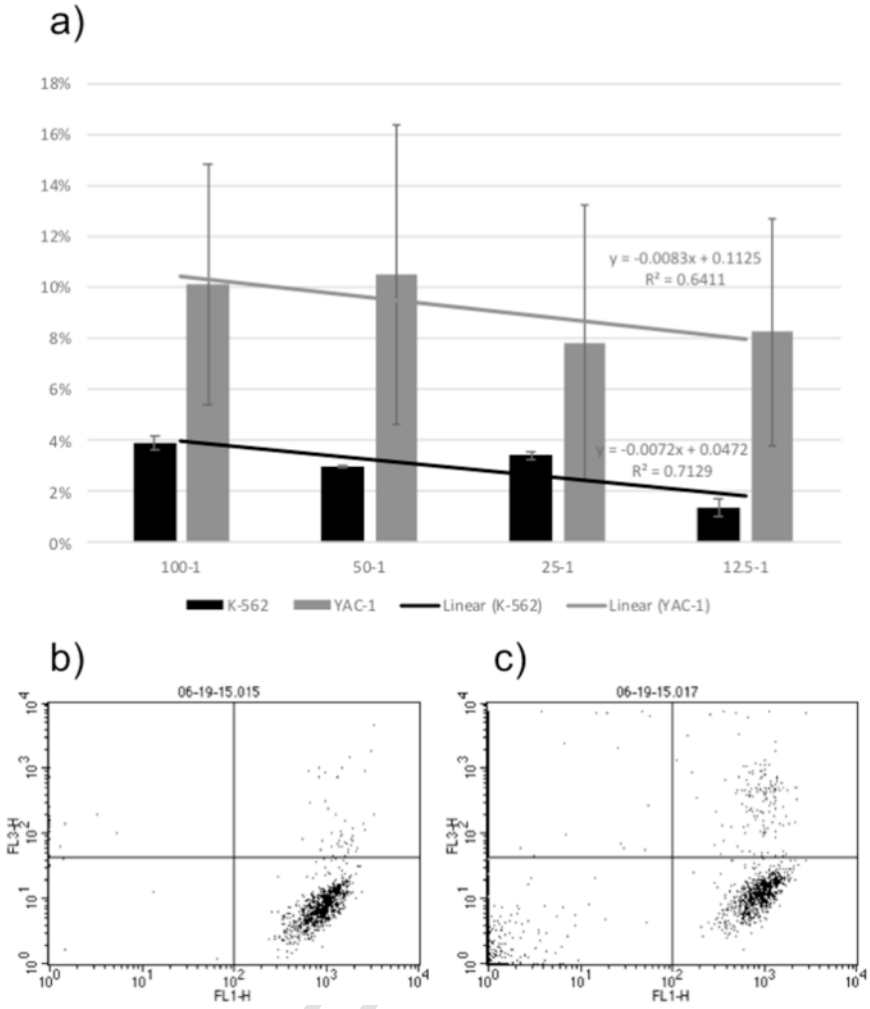

Fig. 1. a). *In vitro* mortality of K-562 (black bars) and YAC-1 (grey bars) after exposure to California sea lion LNMC at different effector: target ratios (100:1; 50:1, 25:1; 12.5:1). Spontaneous mortality was accounted for by adding Propidium Iodide to a tube of YAC-1 cells that lacked effectors, and the result obtained was subtracted to each treatment. Bars indicate ± S.D. Linear regressions with equations and R^2 are shown for each target cell. b) Dot plot demonstrating the labeling of target cells on the X axis (FL1-H) and mortality on the Y axis (FL3-H) in the absence of effector cells (spontaneous mortality), c) Dot plot demonstrating the labeling of target cells on the X axis (FL1-H) and mortality on the Y axis (FL3-H) in the presence of effector cells.

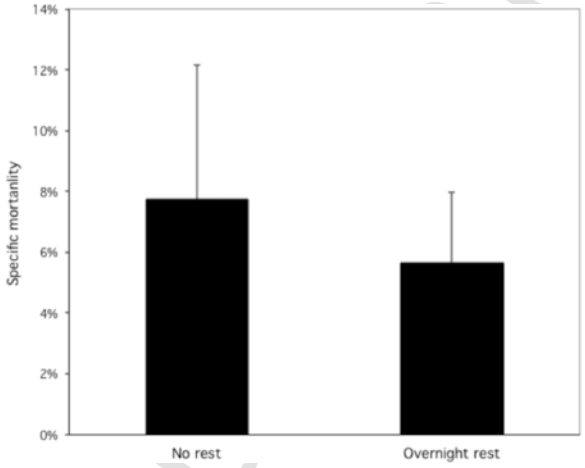

Fig. 2. *In vitro* mortality of YAC-1 cells by California sea lion LNMC with or without an overnight rest, at an effector:target ratio of 50:1. Spontaneous mortality already subtracted. LNMC were extracted at necropsy from 3 animals at the Marine Mammal Center, California. Bars indicate ± S.E.

a concentration-dependent increase in NK cell-like activity for PCB 138 (RM ANOVA; $F_{3,8} = 2.929$, $p = 0.054$; Fig. 3c). The experiment that used the PCB mixture did not show an effect on NK cell-like activity of lymphocytes at any of the experimental concentrations used when compared with 0 ppm (RM ANOVA; $F = 0.458$, $df = 3$, $p = 0.714$; Fig. 3g).

Although results obtained by *in vitro* experiments do not necessarily imply that an organism would respond similarly under natural exposure to contaminants, and effects have been shown to vary between species (Hammond et al., 2005) the environmentally-relevant concentrations used could be considered a good model to understand the effects exerted by organochlorines on CSL NK-cell like and T-cell cytotoxicity, and especially, on oncosurveillance (Canning et al., 2006; Carayannopoulos and Yokoyama, 2004). If the activity of these immune cells is modulated by organochlorines, it is plausible to consider that infection by oncoviruses, such as OtHV-1 (Lipscomb et al., 2000), and tumorigenesis are facilitated in exposed CSL.

3.3. Lymphocyte proliferation

For optimal mitogen concentrations, non dioxin-like congeners 153 (at 5 and 10 ppm) and 180 (at 5 and 10 ppm) and dioxin-like congener 105 (at 5 ppm) showed increased lymphocyte proliferation, and non dioxin-like PCB 138 showed a 43% decrease at the highest (15 ppm) concentration (RM ANOVA; PCB 105, $F_{3,5} = 5.869$, $p = 0.007$; PCB 153, $F_{3,5} = 22.516$, $p < 0.001$; PCB 180, $F_{3,4} = 6.853$, $p = 0.011$; PCB 138, $F_{3,8} = 5.224$, $p = 0.007$; Fig. 4). Although PCB 118 behaved similar to the other congeners analyzed, and showed significant differences among concentrations (RM ANOVA; PCB 118 $F_{3,8} = 3.332$, $p = 0.039$;

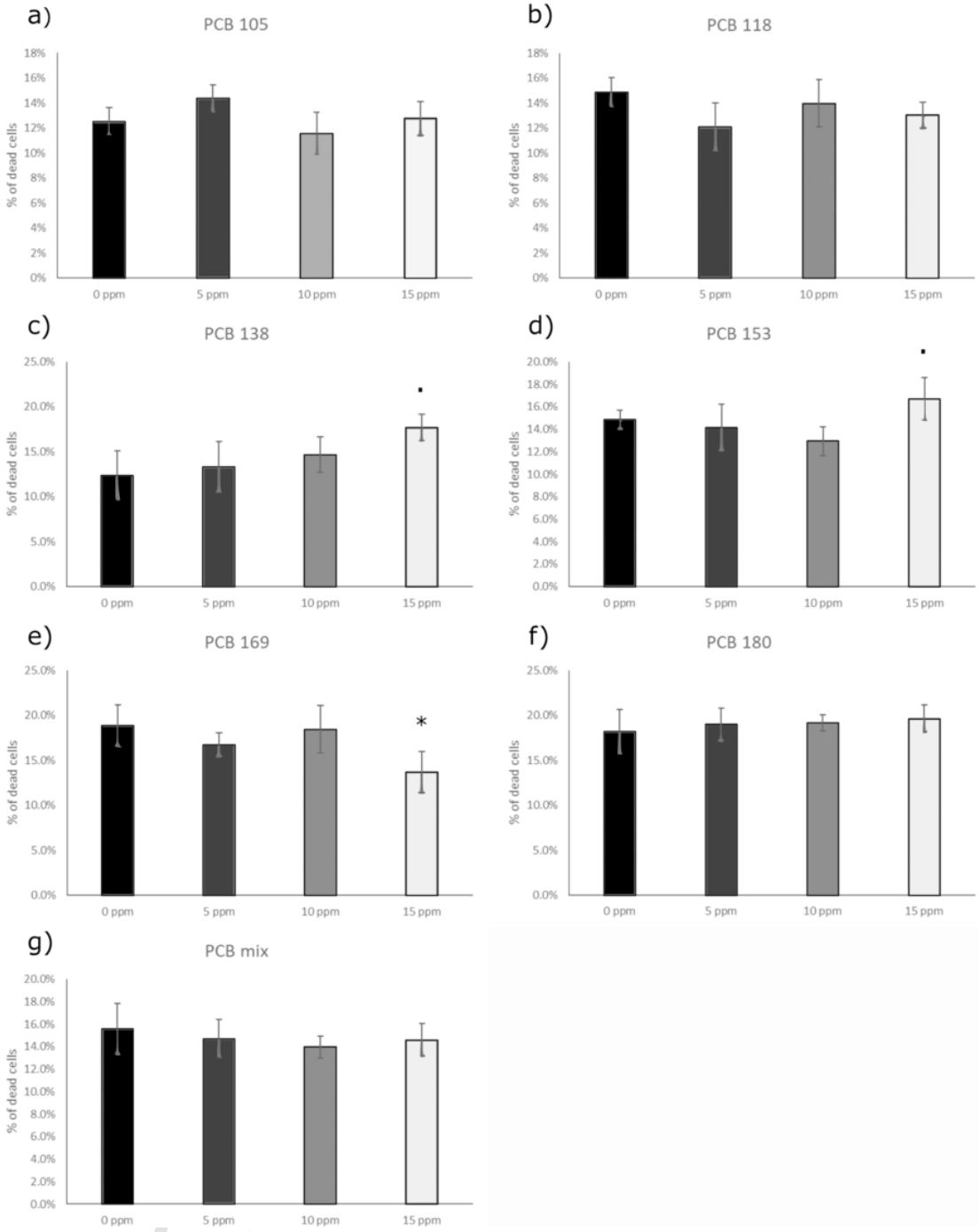

Fig. 3. *In vitro* mortality of YAC-1 cells by California sea lion LNMC exposed to different concentrations of PCB congeners (the effector:target ratio was 50:1). a) PCB 105, b) PCB 118, c) PCB 138, d) PCB 153, e) PCB 169, f) PCB 180, g) PCB mix. Spontaneous mortality already subtracted. LNMC were extracted at necropsy from 8 animals at the Marine Mammal Center, California. Bars indicate ± S.E. * indicates $p < 0.05$,·indicates $p < 0.1$.

Fig. 4b), the *post hoc* Dunnett's test showed no significant difference from unexposed control. PCB 169 did not induce significant changes in lymphocyte proliferation (RM ANOVA; $F_{3,8} = 2.247$, $p = 0.113$; Fig. 4e). Proliferation of lymphocytes exposed to the PCB congener mix at optimal mitogen concentration varied significantly among concentrations (RM ANOVA on RANKS; $Chi^2 = 12.3$, $df = 3$, $p = 0.006$; Fig. 4g), although the *post hoc* test showed no significant difference from unexposed control.

Suboptimal mitogen concentration (Fig. 5), which according to Mori et al. (2006) was considered to be more sensitive to demonstrate immunomodulatory effects in marine mammals, showed similar results to those observed for optimal mitogen concentration. The exception was congener 105, which show a non-significant trend of concentration-dependence (ANOVA on ranks; $Chi^2 = 6.45$, $df = 3$, $p = 0.092$; Fig. 5a). PCB 118 and PCB 169, the other two dioxin-like congeners, showed no difference at suboptimal mitogen concentration (PCB 118, ANOVA on ranks; $Chi^2 = 2.316$, $df = 3$, $p = 0.509$; PCB169, RM ANOVA, $F_{3,7} = 1.170$, $p = 0.339$). Non dioxin-like congeners showed results that were consistent with those found for the optimal mitogen stimulation (RM ANOVA; PCB 138, $F_{3,8} = 2.719$, $p = 0.070$; PCB 153, $F_{3,5} = 22.516$, $p < 0.001$; PCB 180, $F_{3,4} = 6.853$, $p = 0.011$). Although PCB 138 did not vary significantly, the observed pattern was similar to what was found for optimal mitogen concentration; PCB

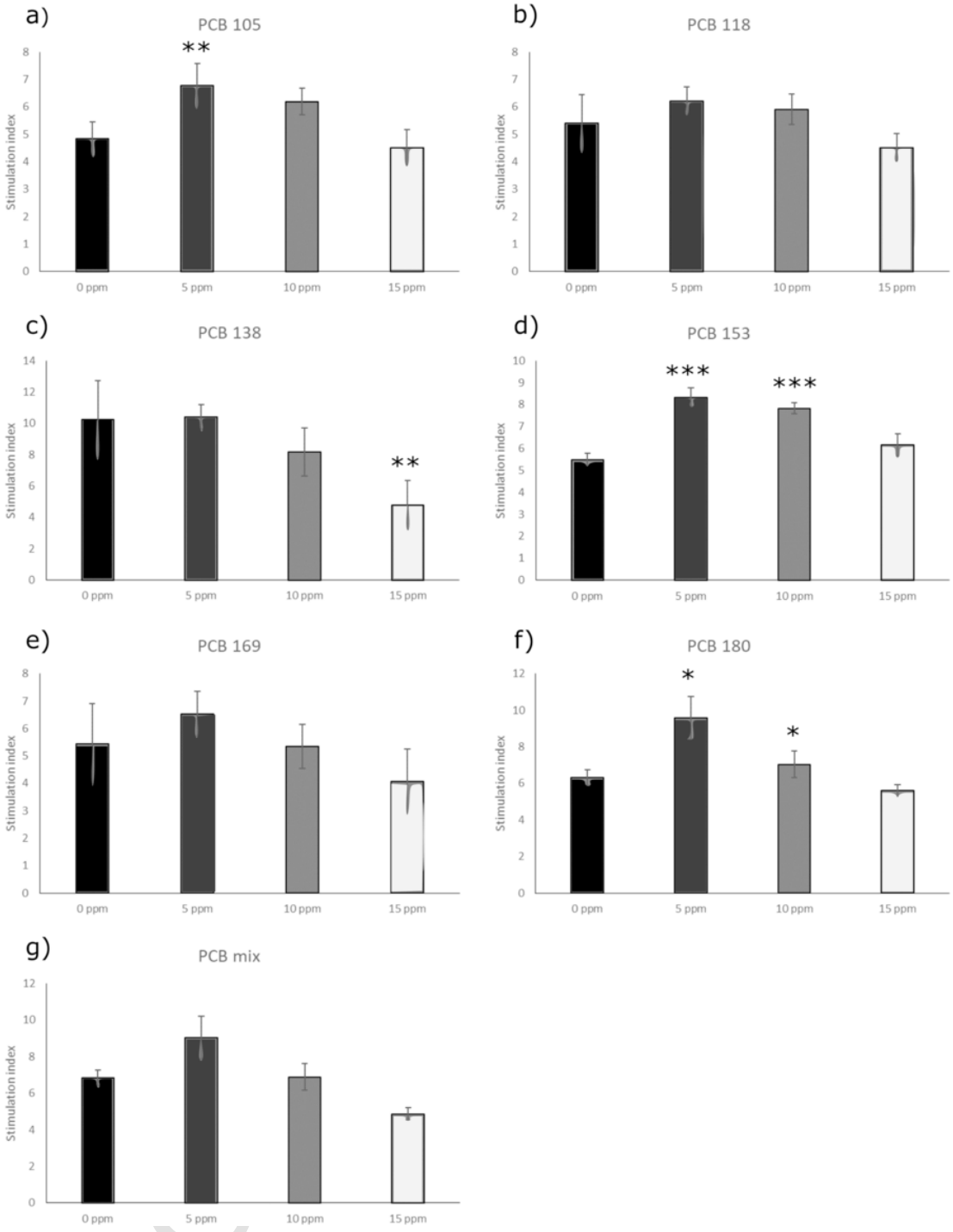

Fig. 4. Proliferation of LNMC (n = 8) exposed to PCB congeners under optimal stimulation (1 μg/mL ConA). a) PCB 105, b) PCB 118, c) PCB 138, d) PCB 153, e) PCB 169, f) PCB 180, g) PCB mix. Proliferation was measured as the Stimulation Index. Bars indicate ± S.E. *** indicates $p < 0.001$, ** indicates $p < 0.01$, *indicates $p < 0.05$.

153 induced differences for both 5 and 10 ppm; and 180 was different at (5 ppm, but not at 10 ppm). Proliferation of lymphocytes exposed to the PCB congener mix at suboptimal mitogen concentration did not show any difference between treatments (RM ANOVA; $F_{3,7} = 1.237$, $p = 0.321$; Fig. 5g). While previous reports suggested higher sensitivity to detect immunomodulatory effects on T lymphocyte proliferation using sub-optimal mitogen concentrations (Mori et al., 2006), this assumption did not hold true in this study, with the sub-optimal mitogen concentration adding the detection of the effects of PCB 105 at 15 ppm, but failed to detect the effects of PCB 105 at 5 ppm and PCB 180 at 10 ppm.

Proliferation varied between both types of PCB congeners. While, except for PCB 138, most of the non dioxin-like congeners appeared to induce proliferation of immune cells when at low concentrations, a clear pattern of concentration-dependent suppression was observed for a dioxin-like congener (PCB 105) when using suboptimal mitogen concentration. It is interesting that a similar effect of PCB 138 was found for East Greenland ringed seal (*Pusa hispida*) at the same concentration as used here (Levin et al., 2016). PCB 138 was found to be able to induce UGT1A6 transcription in rat hepatoma cells independently of the androstane receptor CAB, which is the mechanism by which non dioxin-like PCBs affect this gene transcript. In addition, UTGA1A6 is

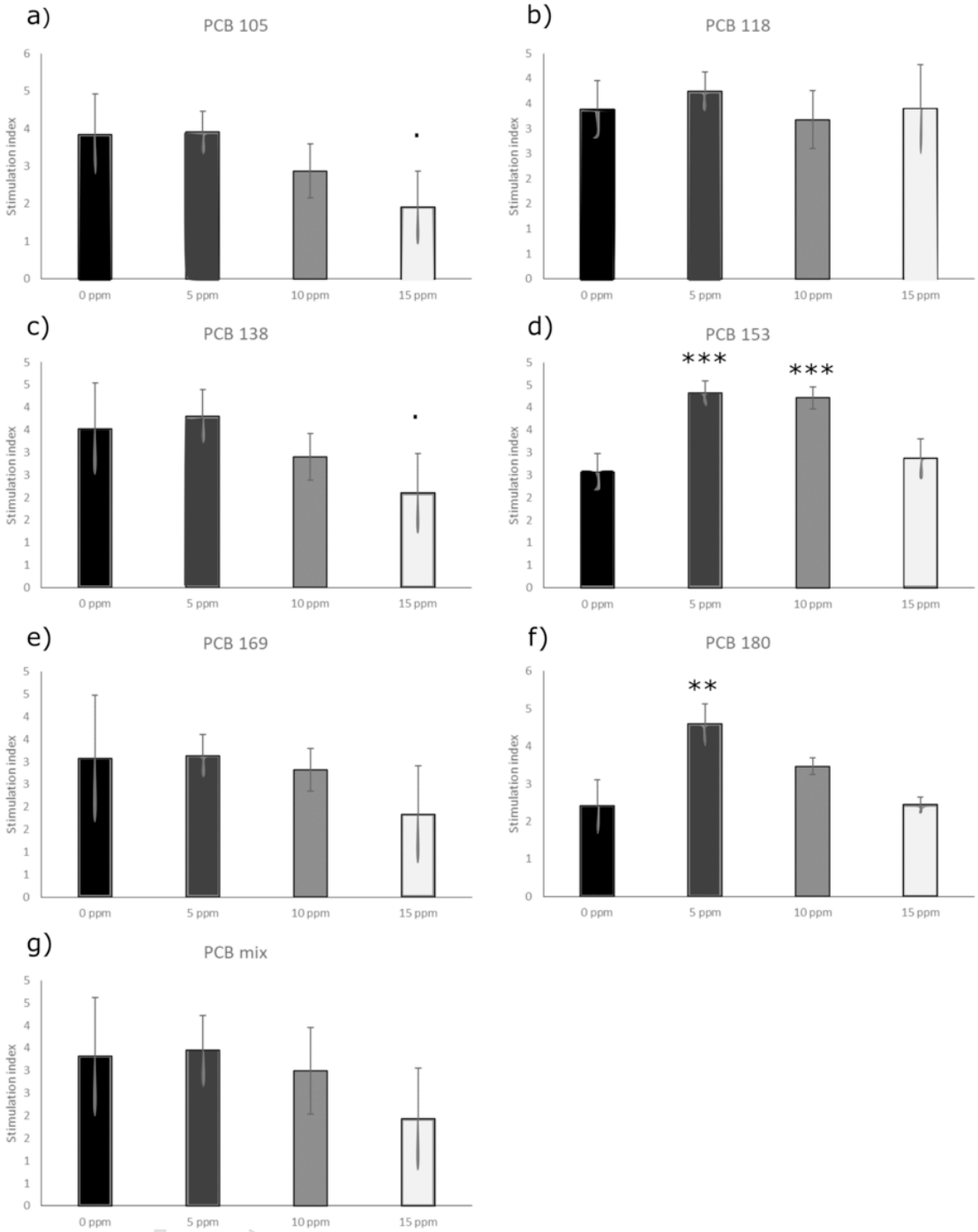

Fig. 5. Proliferation of LNMC exposed to PCB congeners under suboptimal stimulation (0.1 μg/mL ConA). a) PCB 105, b) PCB 118, c) PCB 138, d) PCB 153, e) PCB 169, f) PCB 180, g) PCB mix. Proliferation was measured as the Stimulation Index. LNMC were extracted at necropsy from 8 animals at the Marine Mammal Center, California. Bars indicate ± S.E. *** indicates $p < 0.001$, **.

generally upregulated by the presence of dioxin-like PCBs *via* an AhR-dependent mechanism (Hamers et al., 2011). Although it does not mean that PCB138 is a dioxin-like PCB instead of non dioxin-like, it may exert some structural effect on AhR, or there may be other unexplored cell routes yet to be described. PCB 105 is typically low in free-ranging CSL blubber and serum (Del Toro et al., 2006; Niño-Torres et al., 2009; Ylitalo et al., 2005). These types of low molecular weight, low chlorinated congeners are the first to be mobilized from the blubber to the milk and, thus, become more concentrated in neonatal pups, as has been seen in the grey seal, *Halichoerus grypus* (Sørmo et al., 2003). If so, CSL pup lymphocytes could be restricted in their proliferative ability early in development. More worryingly, dioxin-like PCB congeners can activate the aryl hydrocarbon receptor (AhR) (Denison and Nagy, 2003; Mandal, 2005) and, if persistent, AhR activation could lead to chronic autoimmune processes due to sustained Th17 responses, which are driven by Treg cells, and have been reported to occur in mice (Veldhoen et al., 2008; Huo et al., 2018). In addition, potential negative effects of the PCB congeners on lymphoid organs, such as the thymus or bone marrow, could be enough to drive the individual towards an anergic status (Couillard et al., 2008; Repetto and Baliga, 1997), which could make CSL more susceptible to various pathologies, including urogenital cancer.

It was interesting to notice that the data collected during the assays of CSL LNMC exposed to the PCB mix displayed a markedly non-normal behavior in

terms of distribution and lack of homoscedasticity, both for innate NK cell-like activity and for proliferation at both mitogen concentrations. Plausibly, this behavior could reflect that when exposed to a mixture of dioxin-like and non dioxin-like congeners, CSL LNMC responses can be somewhat unpredictable (Levin et al., 2004, 2005a, 2005b), particularly because of complex additive, antagonistic and synergistic interactions that can occur between planar and non-planar PCBs (Mori et al., 2006). Alternatively, considering that the assays that used dioxin-like congeners 105 and 118 at suboptimal mitogen concentrations also displayed lack of normality and homocedasticity, these congeners could be driving the observed effects of the PCB mixture. Exposure to the mixture of PCB congeners did not significantly affect NK cell-like activity and lymphocyte proliferation at any of the concentrations tested, while individual congeners did, adds to the weight of evidence that the immunomodulatory effects of PCBs in mixtures are not simply additive, as previously suggested (Mori et al., 2006).

Based on the results of this study, it would appear that exposure to environmentally-relevant PCB concentrations either induces proliferation of CSL lymphocytes or does not cause any observable effect. Evidently, we based our experiments on PCB concentrations reported for California sea lion blubber, and it could be discussed that lymphocytes are being exposed to different PCB concentrations when circulating in the blood. However, some studies have shown that plasma and blubber concentrations of persistent organic pollutants are closely correlated in free-ranging bottlenose dolphins, *Tursiops truncatus* (Yordi et al., 2010) and northern elephant seals, M*irounga angustirostris* (Debier et al., 2006), and previous studies have used blubber concentrations of PCBs to assess *in vitro* effects on circulating lymphocytes.

Although our results defy the traditional view of organochlorines as immunosuppressive agents (Desforges et al., 2016; Kerkvliet, 1995; Tryphonas et al., 1991), our findings concur with what has been reported for other pinnipeds (Mori et al., 2006). Organochlorines, particularly non dioxin-like congeners (Mori et al., 2006), could act as modulators of the immune system, and the net result might largely depend on the dosage, the congener(s), and the species in question. This has been observed for phagocytosis in studies that focused on various pinniped species (Levin et al., 2005b) and cetaceans (Levin et al., 2004). Specifically, Steller sea lion, *Eumetopias jubatus*, neutrophils increased phagocytosis when exposed to a combination of dioxin + dioxin-like PCBs, although the other non dioxin-like congeners + dioxin combinations reduced phagocytosis (Levin et al., 2005b). This is why for pinnipeds, the toxic equivalent factors (TEF) calculated for humans and wildlife (Van den Berg et al., 1998) are not good predictors of PCB effects (Mori et al., 2006; Levin et al., 2004, 2005b).

From an environmental perspective, PCB-induced immunomodulation could be of concern as it could drive the immune response to an anergic state in which the exposed cells cannot implement a full response when challenged by a given pathogen, as they have been incompletely stimulated in the past (Mori et al., 2006). The non dioxin-like congeners considered in this work are frequently measured in free-ranging marine mammals (Del Toro et al., 2006; Niño-Torres et al., 2009, 2010; Ylitalo et al., 2005) and some of them (*i.e.* PCB 180) are persistent in the environment due to their highly chlorinated structure (Beyer and Biziuk, 2009). Furthermore, if sea lions are being exposed to pesticides and dioxin-like related compounds during the perinatal stage, it is possible that they are more susceptible to the immunosuppressive effects, as has been suggested for rats (Smialowicz, 2002; Smialowicz et al., 2001) and phocids fed on a diet of polluted herring (Ross et al., 1996b).

4. Conclusions

This study has shown that cryopreserved cells extracted from CSL lymph nodes collected from fresh carcasses display innate NK cell-like activity, and cells remain viable for at least one month after collection. Furthermore, this study presents a model for future toxicology assays of free-ranging freshly dead animals for which collection of blood samples is often not feasible owing to the rapid clotting of blood.

In general, dioxin-like and non dioxin-like PCB congeners exerted different effects on CSL LNMC. Dioxin-like congeners seemed to have a less pronounced effect on proliferation compared to non dioxin-like congeners, similar to what has been reported for other immune effectors of various marine mammals. These congeners had a more noticeable effect at lower concentrations (5 ppm) than at higher concentrations, where the effects tended to disappear. However, in terms of NK cell-like activity, only congener 169 was shown to impact CSL LNMC. Non dioxin-like congeners 153 and 138 had the opposite effect, with a concentration-dependent pattern.

To the best of our knowledge, this is the first time that *in vitro* suppression of NK cell-like cytotoxicity by a dioxin-like congener has been observed in a pinniped, and is also the first time that a non dioxin-like congener (PCB 138) has shown an immunosuppressive effect on *in vitro* lymphocyte proliferation in an otariid pinniped, as was recently reported for a phocid pinniped. The results reported in this study could constitute evidence that the immune system of free-ranging CSL could become modulated by PCBs, making it difficult to respond to challenges, including oncogenic viruses and cell transformation. Further studies should aim to test whether the *in vitro* results herein obtained are observable at the organism level in natural populations.

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