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Effects on Pinniped Immune Response Upon in vitro Exposure to the Perfluorinated Compounds, PFOS and PFOA

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Abstract

This study investigated the effects of environmental toxicants on the immune system of two pinniped species, grey seals (*Halichoerus grypus*) and hooded seals (*Cystophora cristata*). The toxicants included two perfluorinated compounds (PFC), perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), compounds commonly found in a wide variety of household consumer products, including Scotchguard and Teflon. Although corporations such as 3M curtailed the use of these chemicals in the past decade, concentrations of these chemicals are increasing in the arctic aquatic ecosystem and have been measured in the tissues and blood of arctic pinnipeds. However, the effects of these chemicals on the immune system are poorly understood in marine mammals. The hypothesis was tested, "PFOA and PFOS are equally immunotoxic in arctic pinniped species," using the following specific aims, 1) quantify and assess changes in mitogen-induced lymphocyte proliferation upon increasing concentrations of PFOA and PFOS and 2) compare changes in lymphocyte proliferation between two pinniped species. This was the first study to demonstrate the immunotoxic effects of PFOA and PFOS in two pinniped species; however, the effects were not always similar between the species. Importantly, the concentrations tested represent the range found in the blood of free-ranging animals, suggesting that free-ranging animals may be at risk for immunotoxic effects.

Introduction

Perfluorinated compounds

The most widely studied PFCs are PFOA and PFOS, synthetic chemicals used to make fluoropolymer compounds that are incorporated into trademarked products such as Teflon and Scotchgard. PFOA has specific properties such as fire and soil resistance, as well as oil, stain, grease, and water repellency (Perfluorooctanoic Acid, 2012); PFOS is also water- and lipid-repellent (Emerging Contaminants, 2014). These qualities make PFOA and PFOS ideal agents to create non-stick surfaces and waterproof membranes, as well as for use as surfactants in paints and cleaning products (Perfluorooctanoic Acid, 2012). The U.S. Environmental Protection Agency (EPA) states that the routine use of consumer products that are made with Teflon does not pose a health concern to individuals, but the major issue lies in the fact that PFOA and PFOS are very persistent in both humans and the environment and demonstrate the ability to stay in human and animal tissues for long periods of time (Perfluorooctanoic Acid, 2012). Following the release of this information, 3M, the primary manufacturer of PFOS, voluntarily withdrew from PFOS production in 2002 (Emerging Contaminants, 2014).

PFCs in the arctic ecosystem

PFCs are prevalent in the arctic aquatic ecosystem and have been measured in the tissues and blood of arctic marine mammals. For example, several studies have demonstrated the presence of PFCs in the tissues of ringed seals and polar bears in Greenland (Butt et al. 2010, Levin et al., Rigét et al. 2013). Perfluorooctanoic acid

(PFOA) and perfluorooctanesulfonic acid (PFOS) are two common PFCs that have recently been increasing in arctic marine mammals; however, the potential toxic effects of these chemicals on the immune system are poorly understood. This study investigated the immunotoxic effects of PFOA and PFOS in two pinniped species, grey seals (*Halichoerus grypus*) and hooded seals (*Cystophora cristata*).

PFC Exposure

PFCs can enter into the environment through "direct emissions" via the production and disposal of fluorochemicals, or through precursor compounds that yield PFCs upon degradation and serve as "indirect sources" (Butt et al. 2010). Similarly, humans and animals can be directly exposed through ingestion of PFCs in food and water or inhalation via long-range air transport of particulate matter containing PFCs, or through the use of commercial products that eventually break down to PFCs (Emerging Contaminants, 2014). In cases where PFCs are detected in human sera, dietary intake is thought to be the major route of uptake. Contaminated water and soil are the main points of exposure for wildlife, and result in elevated PFC levels in both wildlife and humans (Corsini et al. 2014).

Immunotoxicity

The immune system is composed of many diverse organs and cells that have specialized functions and work cooperatively to protect the host from potentially pathogenic agents including viruses, bacteria, parasites, fungi, neoplastic and non-self cells (Kuby 1997). Immunotoxicity refers to the "ability to detect, quantify, and interpret

direct and indirect alterations of the immune system resulting from exposure to pharmaceuticals, environmental and occupational pollutants, as well as study immune alterations (stimulatory or suppressive) and their mechanisms and effects on susceptibility or duration of infectious, allergic, or autoimmune disease" (Burleson and Dean, 1995). The PFC contaminant-induced immunotoxicity may put humans and wildlife at risk for infection by various pathogens, potentially resulting in morbidity or mortality.

Adverse effects of PFCs in animals

Numerous studies (Butt et al. 2010, Corsini et al. 2014, Levin et al., Rigét et al. 2013) have investigated the effects of PFCs in various species. Research has been conducted to experimentally expose animals to PFOA and PFOS, with various effects including hepatomegaly and hepatic peroxisome proliferation; tumors of the liver, testicles, and pancreas; reproductive and developmental abnormalities including reduced fetal weight, skeletal and cardiac malformations; neurotoxicity; and immunotoxicity (Corsini et al. 2014). In studies that exposed mice to levels of PFOS that are found in the general human population, results showed that antibody production was affected, specifically in suppression of the antigen-specific IgM antibody response (Corsini et al. 2014). Studies in other wildlife species, including birds, turtles, and lizards, have also demonstrated similar immune system effects (Corsini et al. 2014). Taken, together, these data provide the "weight of evidence" that PFCs are toxic to the immune system.

Adverse effects of PFCs in humans

The effects of PFCs on humans have been mounting in, but results are inconclusive and more research is necessary. Potential adverse effects include increased incidence of chronic diseases that have an inflammatory component, such as diabetes, heart disease, and stroke (Corsini et al. 2014). PFCs have also been suggested to interfere with fatty acid metabolism that could serve as a risk factor for metabolic disorders and cardiovascular disease (Corsini et al. 2014). Preliminary data through *in vitro* experiments and field studies involving human populations in close vicinity to PFC plants suggests that PFC exposure may be linked to immunosuppressive effects. Some features of immunity that were affected involved decreased levels of immunoglobulins A and E in females, increased antinuclear antibodies, reduced natural killer cell activity, and reduction of pro-inflammatory cytokine (TNF-α) release. Children with increased serum levels of PFCs may be less receptive to vaccines due to the effects on the immune system's antibody response (Corsini et al. 2014).

Study Rationale

This research project was designed to evaluate the effects of PFOA and PFOS on the immune system, one measure of health, of two pinniped species, grey seals and hooded seals, and to determine if the species tested in the study were equally sensitive to these chemicals. Any modulation of the immune system may increase an individual's susceptibility to bacteria, viruses, fungi, or protozoal agents, resulting in morbidity or mortality. As these chemicals can biomagnify and bioaccumulate in the arctic food chain, the results from this research will contribute in evaluating the consequences of

global pollution and determine how toxicants in the aquatic environments affect arctic marine mammal health. Wildlife health agencies may able to use these data to make scientifically based decisions on the creation of preventative or remedial strategies to protect marine mammals.

Hypothesis and objectives

The hypothesis was tested, “PFOA and PFOS are equally immunotoxic in arctic pinniped species,” using the following specific aims:

1. Quantify and assess changes in mitogen-induced lymphocyte proliferation upon increasing concentrations of PFOA and PFOS,
2. Compare changes in lymphocyte proliferation between pinniped species.

Materials and Methods

Source of cells

The immune cells, including peripheral blood mononuclear cells (PBMC), lymph node lymphocytes (LN), and spleen cells, were acquired by my research advisor, Dr. Milton Levin, in collaboration with the Mystic Aquarium, Sea World, Danish colleagues Rune Dietz and Christian Sonne (Aarhus University, Denmark), and Gordon Waring and Andrea Bogomolni (US Northeast Fisheries Science Center). These cells were harvested and cryopreserved from grey seals and hooded seals under appropriate federal permits and IACUC approval (University of Connecticut).

Functional immune assay

The selected assay for the measurement of the immune response was the mitogen induced lymphocyte proliferation assay, previously demonstrated for marine mammals (Levin et. al, Levin et al. 2010, Levin et al. 2008)). Lymphocyte proliferation is one measure of cell-mediated immunity, important in the generation of effector and memory lymphocytes. Cells were exposed to different mitogens and chemicals, then assessed for proliferation response. In this experiment, the mitogen concanavalin A (ConA) was utilized to stimulate T lymphocytes.

Cryopreserved immune cells were thawed in a 37°C water bath, then transferred to a conical tube with 45 mL complete Dulbecco's Modified Eagle Medium (DMEM) culture medium. The cells were washed three times in complete DMEM at room temperature (25°C) for 10 min at 1000 rpm. Cells were then re-suspended in complete DMEM. A 50 µL aliquot was removed for counting on a hemocytometer, and the cell solution volume was adjusted to achieve a working cell concentration of 2×10^6 cells/ml in complete DMEM. The working solution was plated in triplicate into a 96-well flat bottom microtiter plate. To each well, 100 µL of cells was added, along with 50 µL of chemical at eight increasing concentrations for PFOA (0, 1, 3, 10, 30, 100, 300, and 1000 ppb) or seven increasing concentrations for PFOS (0, 1, 3, 10, 30, 100, and 300 ppb). These concentrations encompass the range of PFOA (0.2 - 3.1 ppb) and PFOS (21.1 - 196.6 ppb) that has been measured in the serum of free ranging East Greenland ringed seals (*Pusa hispida*), another arctic pinniped species (pers comm). For mitogen-stimulated cells, 50 µL of the T cell mitogen ConA, at either one of two concentrations (optimal, 1µg/ml; sub-optimal, 0.1µg/ml) was added to each well, and for the

unstimulated (no mitogen, NM) cells, 50 µL of complete DMEM was added to each well. Plates were incubated for 48 hr at 37°C, 5% CO_2, followed by an additional 18 hr incubation after the addition of 20 µL/well 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog. After incubation, the plate was centrifuged at room temperature for 10 min at 1000 rpm, the supernatant was dumped off, and the plate was allowed to air-dry overnight. Lymphocyte proliferation was detected with a monoclonal antibody and colorimetric enzymatic reaction (Cell Proliferation ELISA BrdU (colorimetric), Roche Diagnostics GmbH, Mannheim Germany) as per manufacturer's instructions using an ELISA plate reader (Multiskan EX v.1.0) at 450 nm with a reference wavelength of 690 nm.

Statistics

A repeated measure one-way analysis of variance (RM-ANOVA) with Dunnett's test was performed to compare the exposed cells to the unexposed cells using $p<0.05$ for statistical significance, as cells from the same individual were divided into several treatment groups. Dose-response curves were generated in Microsoft Excel to visualize the effects of the different concentrations to the unexposed control. Dose response curves were compared between the two species to determine potential species differences.

Although not part of the hypothesis, lymphocyte proliferation of immune cells from three different compartments in the hooded seal, PBMCs, mesenteric lymph nodes, and spleen, was examined to determine if T lymphocytes proliferated at similar rates. A RM-ANOVA with Bonferroni test was performed to compare lymphocyte proliferation among the compartments. In addition, we assessed the effects of PFOA

and PFOS on lymphocytes isolated from lymph nodes. Statistical analysis was performed as per PBMC above. See Appendix A for results.

Results

PFOA and PFOS

Raw values of optical density for lymphocyte proliferation in grey seals and hooded seals with PFOS or PFOA exposure are listed in Tables 1-6.

In vitro exposure of grey seal PBMC to PFOS did not significantly affect lymphocyte proliferation (Figure 1). *In vitro* exposure of grey seal PBMC to PFOA significantly increased lymphocyte proliferation at concentrations of 3, 10, 30, and 100 ppb in cells stimulated with opt-ConA mitogen (Figure 2).

In vitro exposure of hooded seal PBMC to PFOS significantly increased lymphocyte proliferation at concentrations of 3, 10, 30, 100, and 300 ppb in unstimulated cells, and concentrations of 3, 10, 30, and 100 ppb in cells stimulated with opt-ConA and sub-ConA mitogen (Figure 3). *In vitro* exposure of hooded seal PBMC to PFOA significantly increased lymphocyte proliferation at concentrations of 10, 30, and 100 ppb in unstimulated cells, and concentrations of 1, 3, 10, 30, and 100 ppb in cells stimulated with opt-ConA and sub-ConA mitogen (Figure 4).

Discussion

This is the first study to demonstrate the immunotoxic effects of PFOA and PFOS in grey seals and hooded seals. PFOA induced significant effects on grey seal PBMC proliferation only at the optimal ConA concentration, but induced significant effects on

hooded seal PBMC proliferation with both concentrations of ConA, as well as unstimulated cells. PFOS did not have significant effects on grey seal PBMC proliferation; however, the power of the test was low, indicating that a larger sample size is required in order to be confident in negative results. PFOS induced significant effects on hooded seal PBMC proliferation with both concentrations of ConA, as well as unstimulated cells.

Importantly, both PFOA and PFOS induced significant effects on the proliferation of unstimulated hooded seal lymphocytes, indicating that PFOA and PFOS could induce lymphocyte proliferation in the absence of mitogen, but only in hooded seals. It is also important to note that in grey seal PBMC, PFOA induced significant effects only in proliferation of lymphocytes that were stimulated with opt-ConA, which supports the use of ConA at both optimal and sub-optimal concentrations. In the majority of cases of significant effects on proliferation with sub-ConA, there were also significant effects with opt-ConA; however the reverse was not always true. If only the sub-ConA mitogen was used in trial, then we would fail to see significant effects. In future studies, both opt-ConA and sub-ConA concentrations of mitogen should be used.

Immunotoxic risk in wild populations

The concentrations of PFOA and PFOS that were used in this *in vitro* study encompass the range of PFOA (0.2 - 3.1 ppb) and PFOS (21.1 - 196.6 ppb) that has been measured *in vivo* in the serum of free ranging ringed seals, an arctic pinniped species similar to the hooded seals and grey seals included in this study. A comparison between the range of either PFOA or PFOS concentrations found in free ranging

pinnipeds and the *in vitro* concentrations is displayed in Figures 8-11, with the black brackets indicating the range of PFC concentrations in free ranging animals. Although lymphocyte proliferation has been determined to be statistically significant with *in vitro* concentrations of PFOA or PFOS in this study, several *in vitro* concentrations tested fell within the range of *in vivo* concentrations. Therefore, these data suggest that free-ranging pinnipeds may be at risk for the modulation of lymphocyte proliferation induced by PFOA and PFOS. Interestingly, for grey seal PBMCs, exposure to PFOS did not induce any significant changes, even at concentrations observed in free-ranging animals.

Further studies might involve measuring *in vivo* serum concentrations of PFOA and PFOS and observing the correlation with corresponding measured optical densities from the same samples to determine whether the *in vitro* results are accurately representative of the *in vivo* data.

Conclusions

These data reject the hypothesis that PFOA and PFOS are equally immunotoxic in the two pinniped species, grey seals and hooded seals, because the effects on lymphocyte proliferation were not always similar between species. However, the data does provide evidence to support the immunotoxicity of PFOA and PFOS in these species.

Increased lymphocyte proliferation indicates an active immune response against a potentially pathogenic antigen, but the consequences of this response are not fully understood. A modulated level of lymphocyte proliferation is an effective defense

mechanism of the immune system, but uncontrolled proliferation of T cell lymphocytes might lead to neoplastic growth and formation of tumors or lymphomas.

Further studies should be performed to examine B cell lymphocyte proliferation. Uncontrolled proliferation of B cell lymphocytes could lead to overstimulation of the adaptive immune response and might give rise to autoantibodies and subsequent autoimmune disorders.

Appendix A

Comparison among hooded seal immune cells

Figure 7 shows hooded seal lymphocyte proliferation among cells of three different immune compartments, including PBMC, lymph node, and spleen, without exposure to PFOA or PFOS chemicals. Proliferation of PBMC vs. lymph node cells was significantly different only when stimulated with sub-optimal ConA, but there were no other significant differences between the compartments. This study should be expanded with a larger sample size for all three immune compartments in order to strengthen the data and provide for further conclusions. These preliminary data suggest that cells from different immune compartments are not equally proliferative and respond differently to stimulation by ConA mitogen.

In vitro exposure of hooded seal LN to PFOS significantly increased lymphocyte proliferation at a PFOS concentration of 300 ppb in cells stimulated with opt-ConA and sub-ConA mitogen (Figure 5). *In vitro* exposure of hooded seal LN to PFOA significantly increased lymphocyte proliferation at PFOA concentrations of 1, 3, 10, 30,

and 100 ppb for cells stimulated with opt-ConA mitogen and concentrations of 3, 10, 30, 100, and 300 ppb for cells stimulated with sub-ConA mitogen (Figure 6).

Comparison of lymphocyte proliferation among hooded seal PBMC, LN, and spleen

Figure 7 compares lymphocyte proliferation among hooded seal PBMC, LN, and spleen cells at 0 ppb concentration of PFOA chemical. This data has been analyzed separately from the 0ppb concentration of PFOS data, to account for the varied composition of the 0ppb control media (complete DMEM): the media in the PFOS solution contains methanol, but the media in the PFOA solution does not contain methanol.

Table 1. Mitogen induced lymphocyte proliferation values (optical density) for grey seals PBMC upon *in vitro* exposure to increasing concentrations of PFOS.

Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb
NM	Hg 145	0.079	0.072	0.085	0.081	0.089	0.093	0.069
NM	Hg 147	0.086	0.086	0.079	0.092	0.085	0.087	0.084
NM	Hg 149	0.057	0.066	0.065	0.067	0.069	0.088	0.078
NM	Hg 150	0.065	0.067	0.068	0.069	0.074	0.080	0.078
NM	Hg 152	0.043	0.047	0.048	0.055	0.050	0.046	0.048
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb
opt ConA	Hg 145	0.393	0.378	0.497	0.500	0.746	0.587	0.191
opt ConA	Hg 147	0.318	0.333	0.318	0.376	0.322	0.332	0.333
opt ConA	Hg 149	0.665	0.821	0.876	0.978	0.889	0.915	0.763
opt ConA	Hg 150	0.471	0.442	0.530	0.460	0.479	0.475	0.401
opt ConA	Hg 152	0.057	0.063	0.063	0.065	0.063	0.066	0.064
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb
sub ConA	Hg 145	0.124	0.143	0.143	0.146	0.150	0.158	0.083
sub ConA	Hg 147	0.132	0.141	0.133	0.158	0.176	0.131	0.116
sub ConA	Hg 149	0.449	0.630	0.687	0.748	0.645	0.697	0.399
sub ConA	Hg 150	0.216	0.214	0.297	0.241	0.327	0.273	0.187
sub ConA	Hg 152	0.053	0.055	0.060	0.063	0.059	0.061	0.061

Table 2. Mitogen induced lymphocyte proliferation values (optical density) for grey seals PBMC upon *in vitro* exposure to increasing concentrations of PFOA.

Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb	1000 ppb
NM	Hg 145	0.094	0.091	0.085	0.094	0.094	0.088	0.087	0.100
NM	Hg 141	0.153	0.100	0.085	0.095	0.073	0.079	0.072	0.072
NM	Hg 149	0.064	0.065	0.067	0.071	0.072	0.068	0.071	0.071
NM	Hg 150	0.059	0.059	0.065	0.064	0.067	0.069	0.060	0.072
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb	1000 ppb
opt ConA	Hg 145	0.442	0.605	0.605	0.626	0.719	0.634	0.551	0.504
opt ConA	Hg 141	0.630	0.721	0.830	1.046	0.893	0.859	0.727	0.603
opt ConA	Hg 149	0.793	0.841	1.044	1.045	1.059	0.999	0.943	0.774
opt ConA	Hg 150	0.330	0.382	0.459	0.368	0.358	0.491	0.372	0.368
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb	1000 ppb
sub ConA	Hg 145	0.154	0.161	0.170	0.182	0.159	0.186	0.195	0.179
sub ConA	Hg 141	0.414	0.417	0.346	0.446	0.401	0.421	0.366	0.247
sub ConA	Hg 149	0.591	0.671	0.696	0.784	0.763	0.696	0.639	0.541
sub ConA	Hg 150	0.152	0.189	0.215	0.215	0.214	0.226	0.191	0.161

Table 3. Mitogen induced lymphocyte proliferation values (optical density) for hooded seals PBMC upon *in vitro* exposure to increasing concentrations of PFOS.

Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb
NM	K1	0.100	0.126	0.131	0.123	0.137	0.145	0.160
NM	K4	0.085	0.091	0.095	0.092	0.103	0.110	0.100
NM	K7	0.109	0.146	0.138	0.148	0.143	0.141	0.151
NM	K2	0.101	0.098	0.101	0.104	0.100	0.115	0.124
NM	K3	0.060	0.068	0.072	0.071	0.074	0.065	0.057
NM	K8	0.088	0.097	0.098	0.106	0.107	0.112	0.118
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb
opt ConA	K1	1.970	2.054	2.319	2.292	2.250	2.219	2.129
opt ConA	K4	1.851	1.858	1.923	1.989	2.001	1.818	1.566
opt ConA	K7	2.228	2.236	2.249	2.308	2.267	2.369	2.050
opt ConA	K2	1.781	2.013	2.047	2.052	1.941	1.994	1.875
opt ConA	K3	1.810	1.781	2.035	2.126	2.035	1.914	1.524
opt ConA	K8	1.431	1.694	1.845	1.881	1.802	1.768	1.579
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb
sub ConA	K1	2.007	1.941	2.075	1.981	2.120	1.939	1.851
sub ConA	K4	1.614	1.767	1.699	1.933	1.903	1.696	1.394
sub ConA	K7	1.795	1.924	1.923	1.883	1.964	1.836	1.617
sub ConA	K2	1.293	1.515	1.724	1.725	1.713	1.666	1.148
sub ConA	K3	1.065	1.238	1.467	1.532	1.456	1.338	1.130
sub ConA	K8	1.093	1.407	1.538	1.473	1.487	1.506	1.451

Table 4. Mitogen induced lymphocyte proliferation values (optical density) for hooded seals PBMC upon *in vitro* exposure to increasing concentrations of PFOA.

Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb	1000 ppb
NM	K1	0.097	0.084	0.091	0.100	0.100	0.096	0.087	0.084
NM	K4	0.089	0.115	0.097	0.105	0.099	0.101	0.102	0.084
NM	K7	0.169	0.184	0.160	0.166	0.164	0.183	0.155	0.143
NM	K2	0.081	0.112	0.121	0.128	0.130	0.150	0.126	0.114
NM	K3	0.058	0.063	0.068	0.070	0.072	0.068	0.063	0.055
NM	K8	0.091	0.123	0.138	0.153	0.156	0.129	0.125	0.091
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb	1000 ppb
opt ConA	K1	1.766	1.945	2.058	2.094	2.024	2.118	1.918	1.721
opt ConA	K4	1.808	1.835	2.047	1.948	1.988	1.848	1.808	1.663
opt ConA	K7	2.110	2.200	2.022	2.277	2.128	2.105	2.010	2.138
opt ConA	K2	1.426	1.655	1.749	1.777	1.835	1.723	1.642	1.507
opt ConA	K3	1.303	1.531	1.623	1.743	1.611	1.563	1.331	1.021
opt ConA	K8	1.499	1.747	1.730	1.751	1.887	1.842	1.819	1.702
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb	1000 ppb
sub ConA	K1	1.676	1.837	1.957	1.979	1.970	1.996	1.687	1.745
sub ConA	K4	1.580	1.944	1.928	1.826	1.915	1.721	1.722	1.603
sub ConA	K7	1.949	1.964	1.984	1.860	1.969	1.743	1.666	1.668
sub ConA	K2	1.356	1.570	1.641	1.567	1.708	1.599	1.431	1.203
sub ConA	K3	1.050	1.200	1.305	1.404	1.378	1.255	1.077	0.859
sub ConA	K8	1.406	1.488	1.609	1.563	1.593	1.561	1.559	1.526

Table 5. Mitogen induced lymphocyte proliferation values (optical density) for hooded seals LN upon *in vitro* exposure to increasing concentrations of PFOS.

Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb
NM	K1	0.167	0.129	0.133	0.139	0.124	0.118	0.129
NM	K4	0.090	0.067	0.069	0.067	0.082	0.077	0.075
NM	K7	0.135	0.106	0.108	0.107	0.108	0.105	0.118
NM	K2	0.097	0.115	0.099	0.098	0.098	0.082	0.095
NM	K3	0.075	0.089	0.077	0.086	0.081	0.109	0.098
NM	K8	0.126	0.132	0.164	0.131	0.158	0.152	0.130
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb
opt ConA	K1	1.411	1.416	1.570	1.518	1.389	1.431	1.349
opt ConA	K4	0.926	1.022	1.052	0.999	1.006	0.914	0.618
opt ConA	K7	1.866	1.837	1.786	1.721	1.481	1.478	1.392
opt ConA	K2	1.006	1.150	1.125	1.158	1.052	0.897	0.592
opt ConA	K3	0.909	0.823	0.889	0.989	0.908	1.004	0.907
opt ConA	K8	1.047	1.218	1.179	1.240	1.308	1.213	0.874
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb
sub ConA	K1	0.997	1.097	1.068	1.140	1.095	1.023	0.937
sub ConA	K4	0.476	0.476	0.537	0.511	0.474	0.418	0.275
sub ConA	K7	1.112	1.191	1.216	1.322	1.069	1.167	0.948
sub ConA	K2	0.387	0.456	0.512	0.398	0.467	0.350	0.234
sub ConA	K3	0.805	0.849	0.701	0.738	0.807	0.662	0.658
sub ConA	K8	1.076	1.066	1.120	1.098	1.124	1.008	0.751

Table 6. Mitogen induced lymphocyte proliferation values (optical density) for hooded seals LN upon *in vitro* exposure to increasing concentrations of PFOA.

Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb	1000 ppb
NM	K1	0.129	0.091	0.105	0.098	0.115	0.098	0.094	0.112
NM	K4	0.080	0.092	0.104	0.100	0.095	0.086	0.087	0.074
NM	K7	0.097	0.122	0.128	0.119	0.108	0.101	0.105	0.104
NM	K2	0.111	0.130	0.131	0.112	0.121	0.116	0.114	0.082
NM	K3	0.076	0.074	0.076	0.088	0.092	0.084	0.071	0.059
NM	K8	0.107	0.103	0.133	0.123	0.118	0.129	0.103	0.109
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb	1000 ppb
opt ConA	K1	1.659	1.782	1.876	1.716	1.635	1.606	1.597	1.374
opt ConA	K4	1.274	1.632	1.444	1.479	1.399	1.441	1.346	1.347
opt ConA	K7	1.472	1.790	1.798	1.862	1.946	1.806	1.625	1.696
opt ConA	K2	0.958	0.984	1.294	1.219	1.218	1.200	1.057	0.826
opt ConA	K3	0.939	1.182	1.189	1.321	1.283	1.259	1.355	0.953
opt ConA	K8	1.026	1.274	1.287	1.366	1.365	1.468	1.290	1.187
Mitogen	Animal ID	0 ppb	1 ppb	3 ppb	10 ppb	30 ppb	100 ppb	300 ppb	1000 ppb
sub ConA	K1	1.177	1.127	1.478	1.222	1.319	1.377	1.253	0.945
sub ConA	K4	0.669	0.818	0.777	0.841	1.189	0.908	0.719	0.742
sub ConA	K7	1.040	1.072	1.155	1.282	1.328	1.195	1.163	0.996
sub ConA	K2	0.481	0.433	0.565	0.627	0.681	0.622	0.512	0.463
sub ConA	K3	0.618	0.831	0.986	0.923	1.038	0.955	0.916	0.667
sub ConA	K8	0.791	0.951	0.957	1.130	1.133	1.157	1.084	0.969

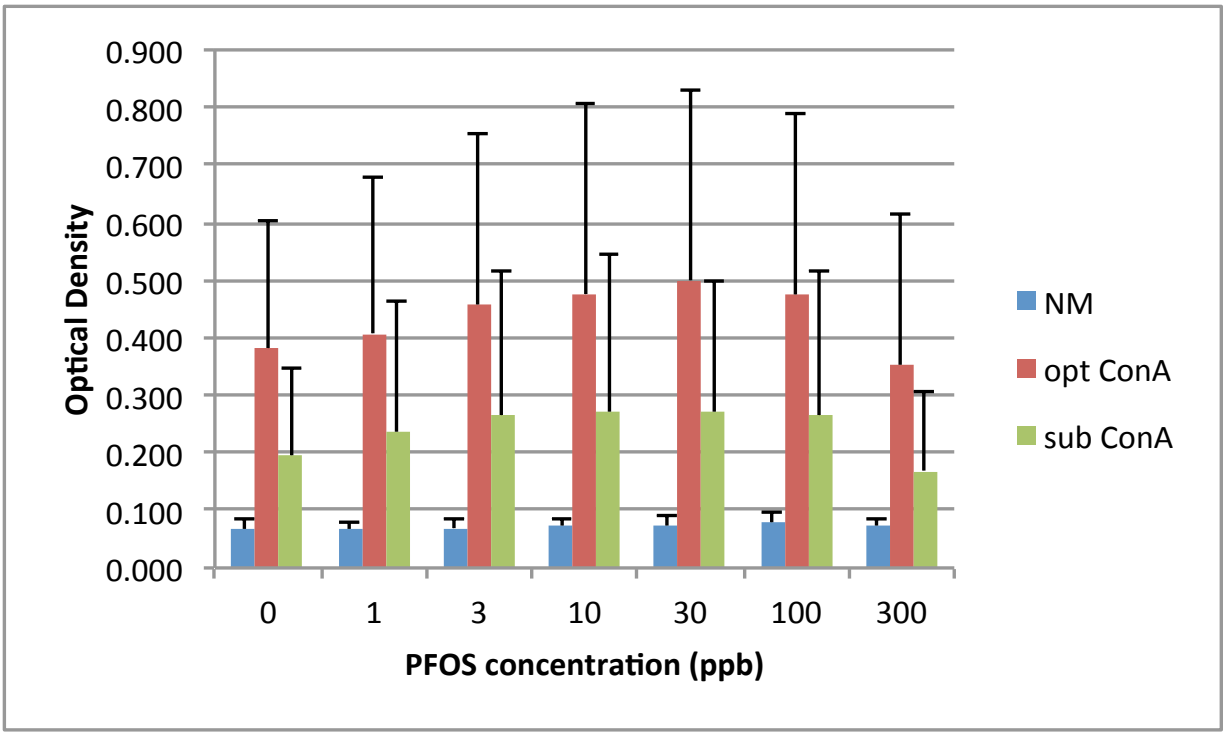

Figure 1. Effects of PFOS on lymphocyte proliferation in grey seal PBMC (n=5). No significant findings.

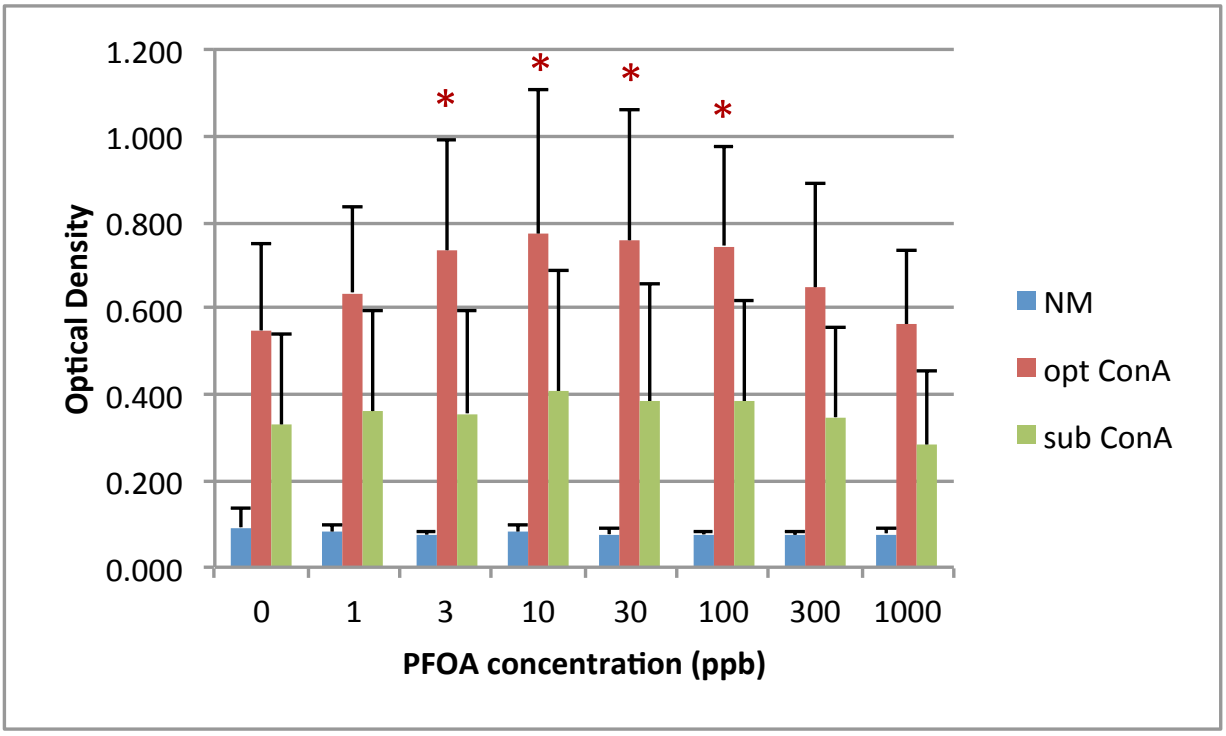

Figure 2. Effects of PFOA on lymphocyte proliferation in grey seal PBMC (n=4). *, $p<0.05$ compared to 0 ppb.

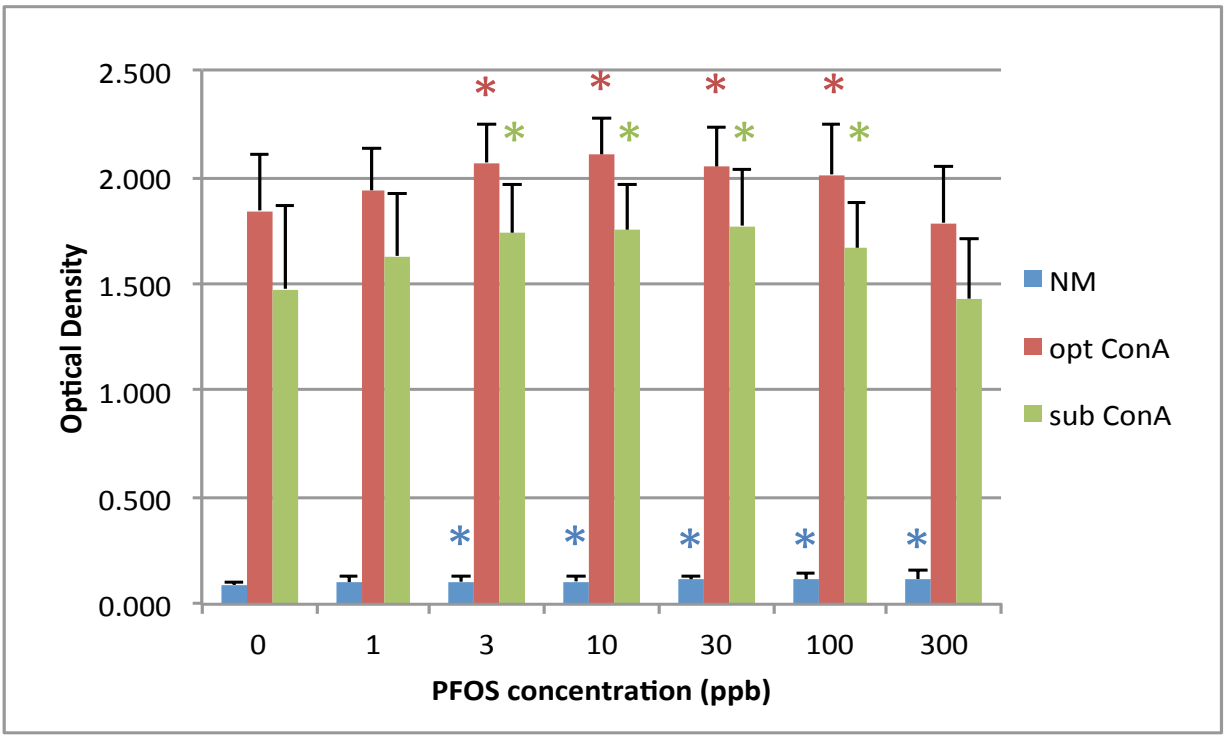

Figure 3. Effects of PFOS on lymphocyte proliferation in hooded seal PBMC (n=6). *, p< 0.05 compared to 0 ppb.

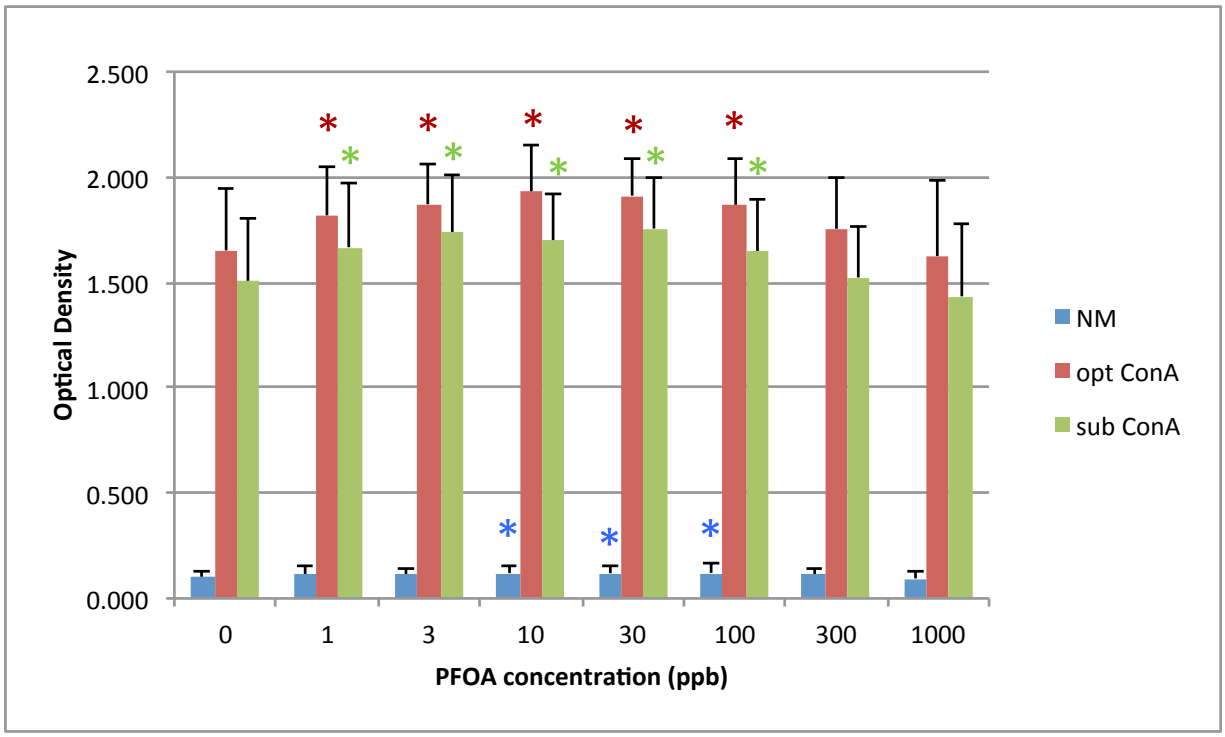

Figure 4. Effects of PFOA on lymphocyte proliferation in hooded seal PBMC (n=6). *, p< 0.05 compared to 0 ppb.

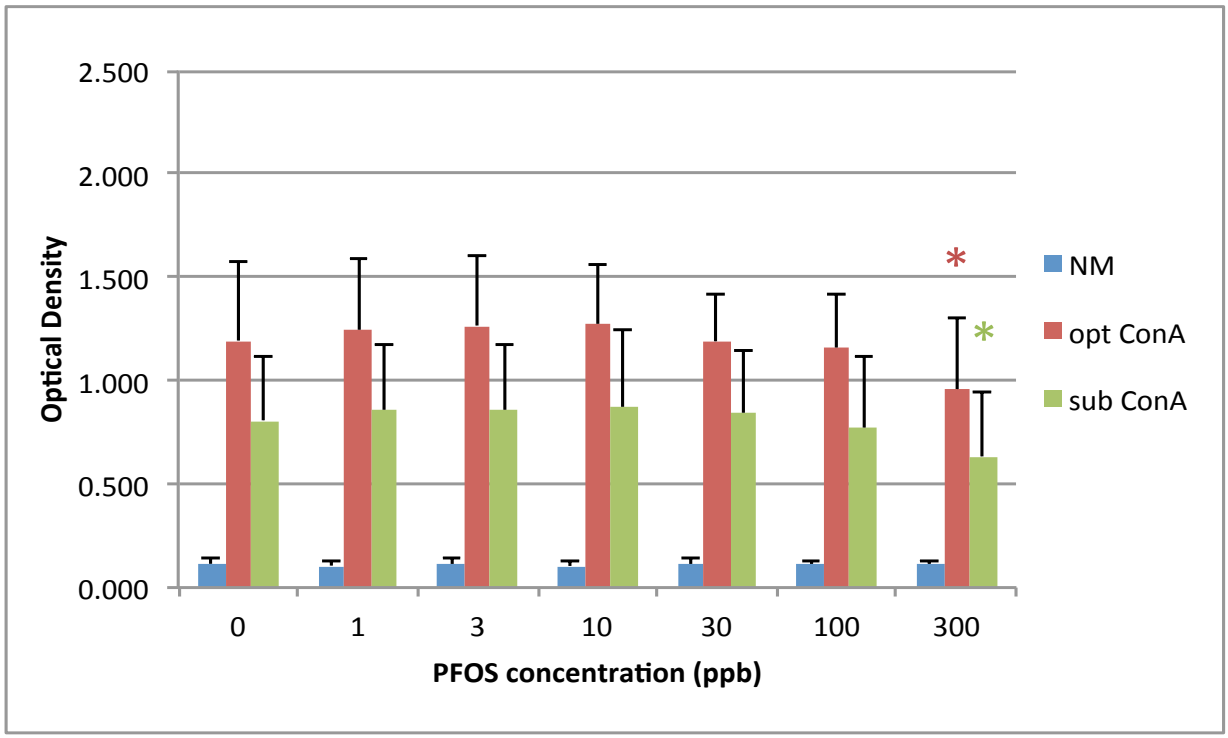

Figure 5. Effects of PFOS on lymphocyte proliferation in hooded seal LN (n=6).
*, p< 0.05 compared to 0 ppb.

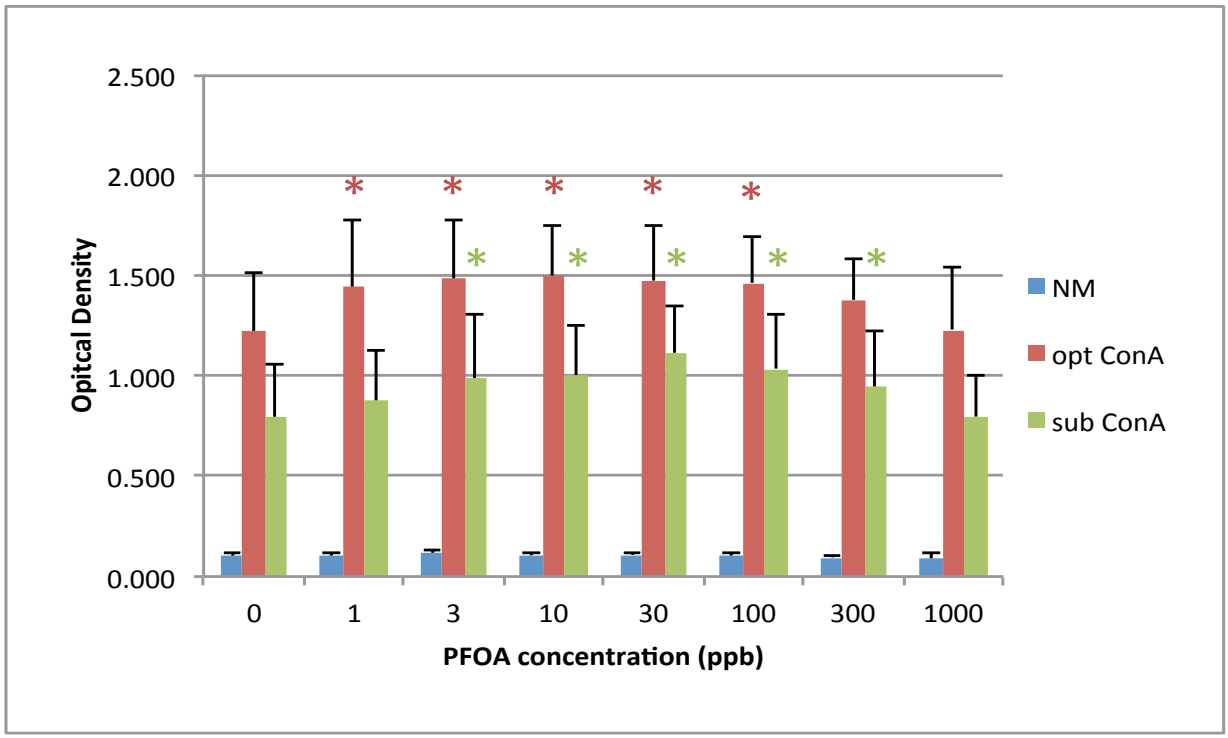

Figure 6. Effects of PFOA on lymphocyte proliferation in hooded seal LN (n=6).
*, p< 0.05 compared to 0 ppb.

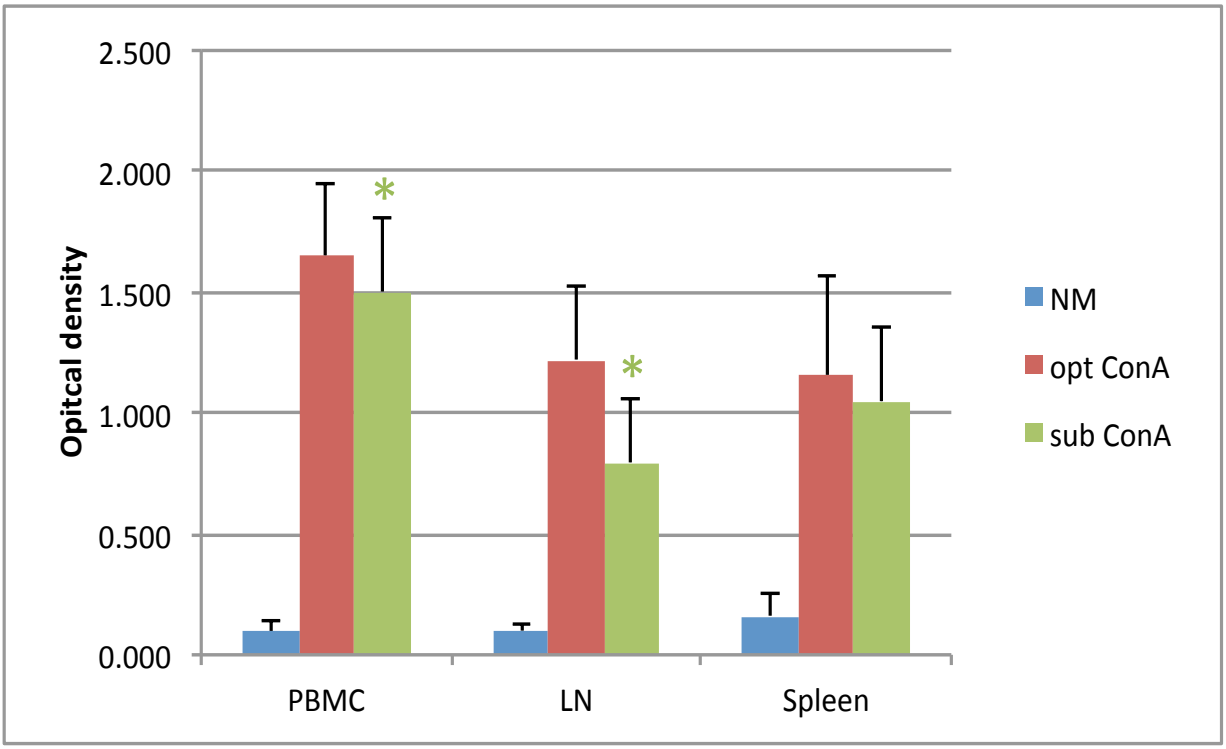

Figure 7. Comparison of lymphocyte proliferation among hooded seal PBMC, LN, and spleen cells (no chemical, 0 ppb PFOA). PBMC, (n=6). LN, (n=6). Spleen, NM (n=6), opt ConA & sub ConA (n=4). *, $p < 0.05$ compared between PBMC and LN with sub ConA mitogen.

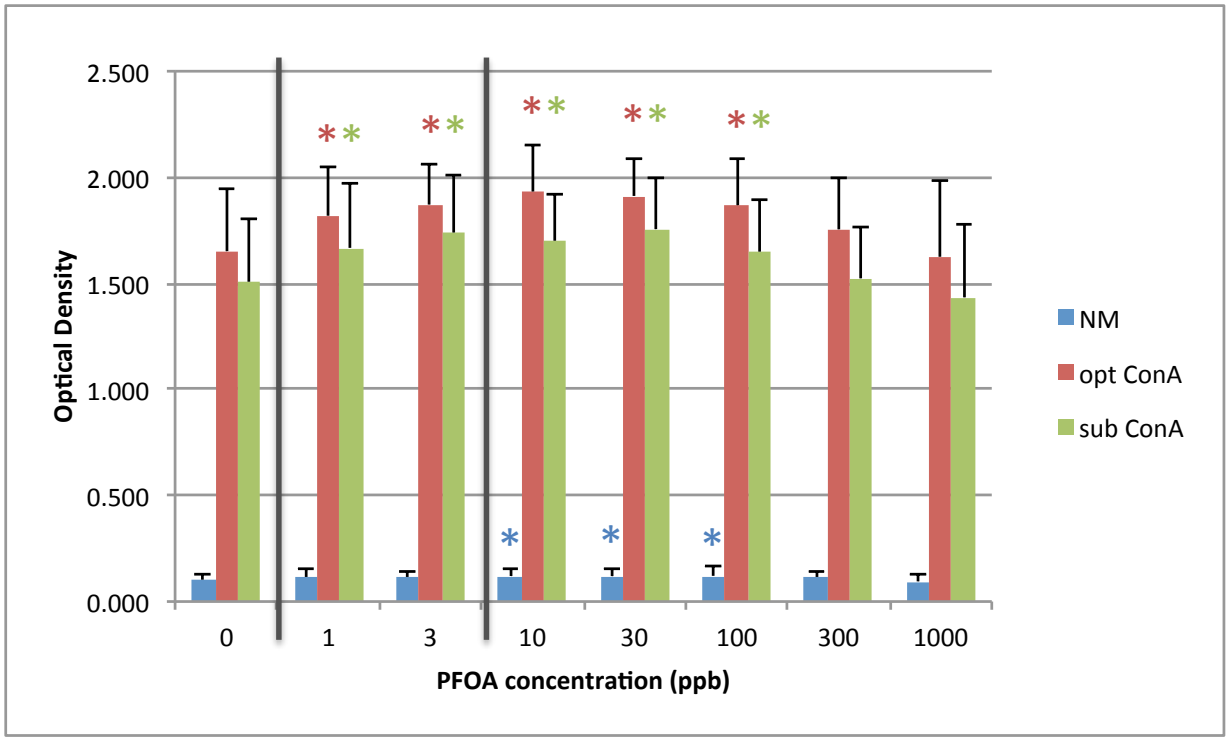

Figure 8. Comparison of the range of PFOA concentration found in free ranging pinnipeds (black brackets) with *in vitro* hooded seal PBMC lymphocyte proliferation under PFOA exposure. *, $p < 0.05$ compared to 0 ppb.

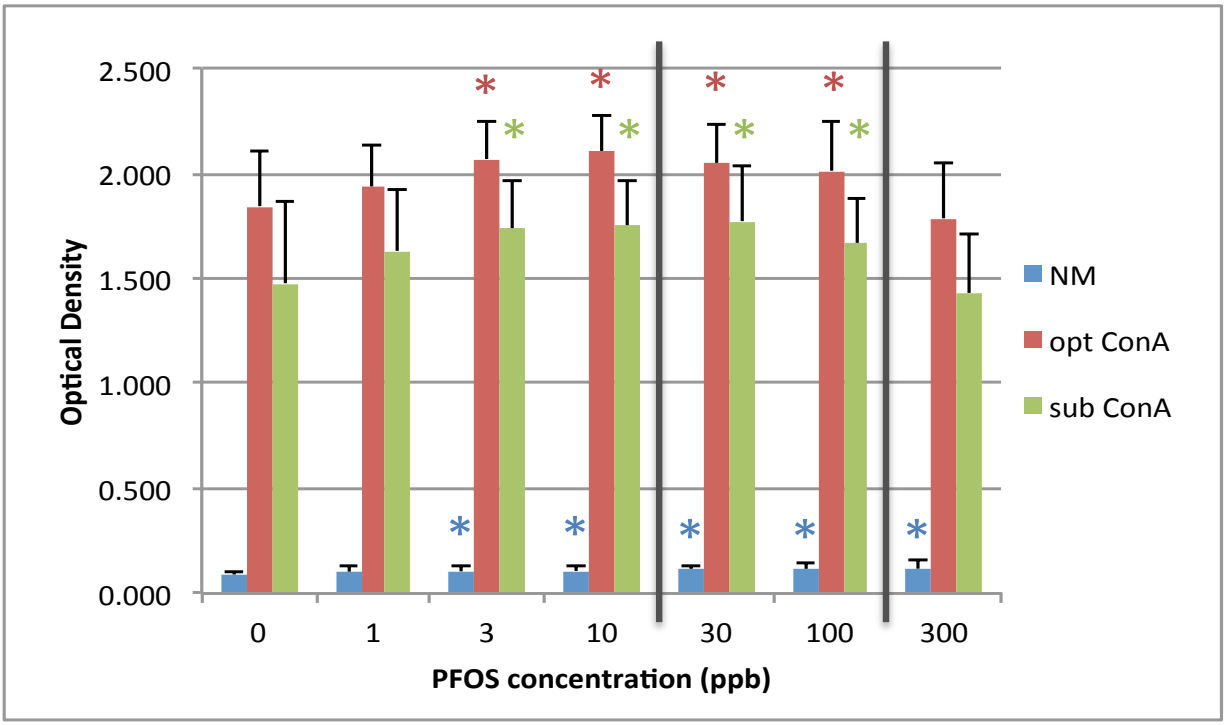

Figure 9. Comparison of the range of PFOS concentration found in free ranging pinnipeds (black brackets) with *in vitro* hooded seal PBMC lymphocyte proliferation under PFOS exposure. *, $p < 0.05$ compared to 0 ppb.

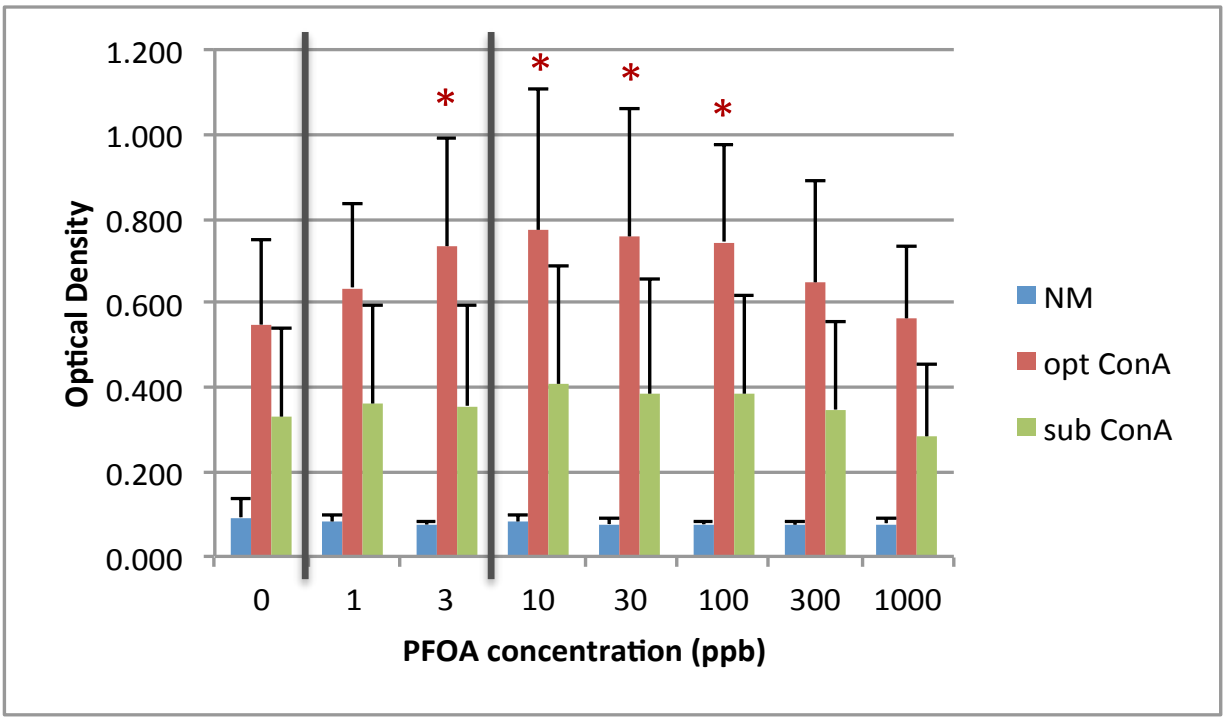

Figure 10. Comparison of the range of PFOA concentration found in free ranging pinnipeds (black brackets) with *in vitro* grey seal PBMC lymphocyte proliferation under PFOA exposure. *, $p < 0.05$ compared to 0 ppb.

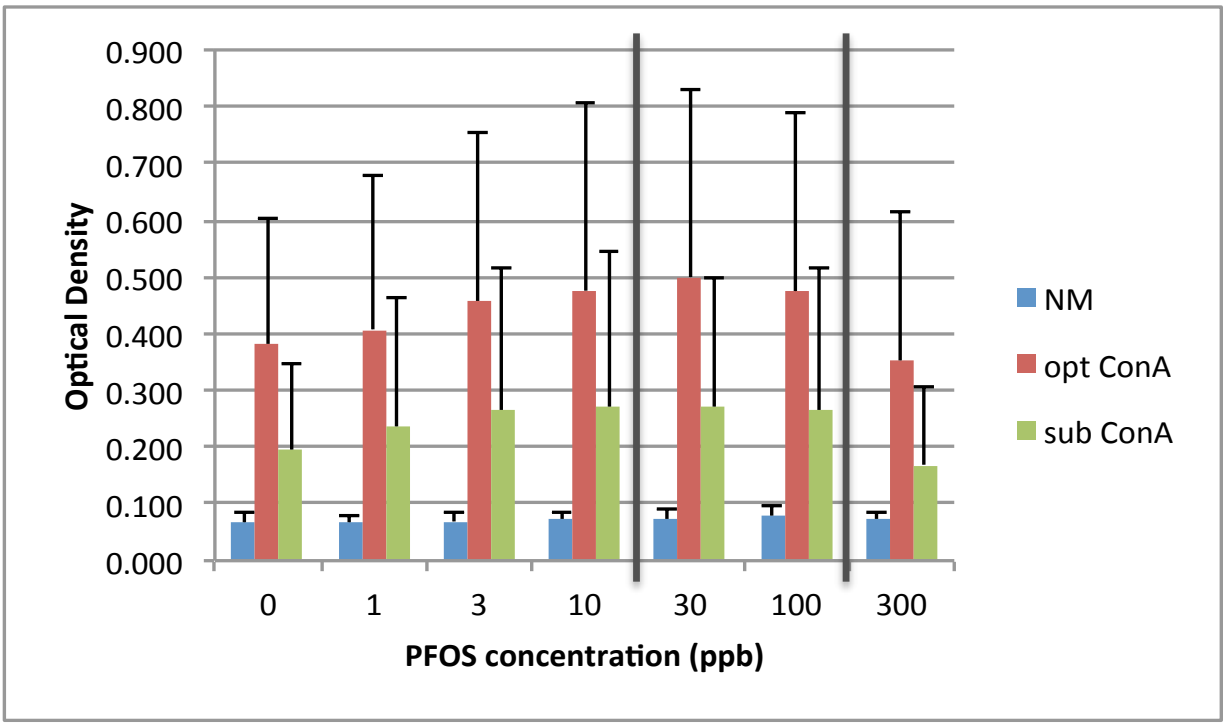

Figure 11. Comparison of the range of PFOS concentration found in free ranging pinnipeds (black brackets) with *in vitro* grey seal PBMC lymphocyte proliferation under PFOS exposure. *, p< 0.05 compared to 0 ppb.

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