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Institut National de la Recherche Scientifique
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Immunotoxic Impact of Pharmaceuticals on Harbor Seal (*Phoca vitulina*) Leukocytes

Par

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Thèse présentée pour l'obtention
du grade de Philosophiae doctor (Ph.D.) en Biologie

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This thesis is dedicated to the seals of Eastern Canada.

I love you dearly.

ACKNOWLEDGEMENTS

First and foremost, none of this work would have been possible without the financial support of the *Institut National de la Recherche Scientifique-Institut Armand-Frappier* and the *Canada Research Chair in Immunotoxicology* (obtained by Prof. Michel Fournier). I was personally also supported by the *German Academic Exchange Service (DAAD)* and the *Fondation Armand-Frappier* with one-year scholarships and would like to thank these organizations.

Secondly, I would like to thank my supervisors Michel Fournier and Sylvain De Guise. Thank you, Michel, for giving me the freedom to develop my own project, and follow my personal research interest. I was able to grow and develop my independence as a researcher in your laboratory. Thank you also for financing my attendance in nine national and international conferences. The exchange with other researchers in the field was an invaluable asset. Thank you, Sylvain, for your help in shaping my articles to their current form. I highly valued and appreciated your input and am grateful that you pushed me to be a better writer.

Furthermore, I would like to thank the members of the jury Cathy Vaillancourt, François Gagné, Daniel Martineau and André Lajeunesse to have accepted to correct and evaluate my thesis. Some of you have accompanied me through more than this last exam and helped shape my project to what it is today. Thank you for your continued interest in my research and guidance.

Thank you to Mike Hammill and the Marine Mammal group of the Institut Maurice-Lamontagne for the grey seal muscle samples and for letting me hitchhike during your field work. A special thanks to the field team of 2014 including Pierre Carter, Joanie Van De Walle and Caroline Sauvé for making 13 h work days fun and relaxing. I would like to take this moment to apologize to the harbor seals of the Bic National Park and Métis. But thank you for providing me with your blood cells. I highly appreciate it.

I would like to thank Stéphane Masson from the Aquarium du Québec and Stéphane Lair for their help in obtaining blood samples from the four harbor seal residents of the aquarium. And thanks to Dalia, Cléo, Daphnée and Nikki for some of your blood. Sorry that my research was an inconvenience for you.

Moreover, I would like to thank Sébastien Sauvé for his interest in collaborating with me to determine if seals do accumulate APIs. It is bittersweet that we were not able to finish the

project, but I am happy that it might be finalized with one of your interns. Thank you also to Sung Vo Duy for help with technical questions and for your time. Furthermore, I'd like to thank David Chatenet for letting me utilize the freeze-dry system of your lab and to Myriam Létourneau for technical assistance. I'm sorry for the smelly samples!

Thanks to Patrick Labonté and his team for letting me use some of their space and material every once in a while.

A big thank you to Marlène Fortier, the fairy godmother of our lab, for teaching me everything I know about the FACS, your technical assistance and personal support in times of need. Thanks also to Pauline Brousseau for our discussions and for your help in correcting especially my earlier pieces of writing for exams. Thank you for welcoming me in your home during the field work period. I really enjoyed our table conversations.

The past four years would not have been the same without my fellow labmates Dr. Émilie Lacaze, Dr. Lauris Evariste, Dr. Julie Pédelucq, Dr. Marc Fraser, Dr. Messika Revel, Zohra Omori, Philippine Granger, Alexandre Beaudry et Pierre-Luc Cloutier. You will be in my heart forever and I hope we will see each other again in future congresses. A special thanks to my two interns Méryl Mounier and João Victor Alvaia and my Apprentis Bruno Fernandez Anaya, Kimberly Gale and Yoan Patry-Menard. Teaching hands-on science for the first time was a very gratifying experience with you.

Not a thanks, but a heartfelt sorry, to my friends from Germany for not having written more in the past couple of years. I am confident this will change now. And a huge thanks to my family for being there for me, and for your sacrifices that allowed me to be where I am today.

Lastly, I'd like to thank my Canadian family Matthieu Blanchet and Pamela H. D. Kleinert. Matthieu, not a thousand words can describe how grateful I am for your existence. You fought and suffered through this project just as hard as I did. When results were coming in you were just as happy and relieved as I was. We rode the emotional rollercoaster of scientific research together. And Pamela, even though you have no idea what was happening in the past 2 ½ years, thank you for bringing a light and many laughs into our lives. But please, in the future, let us sleep past 4 a.m., if it's possible. Thanks.

Der Panther

Sein Blick ist vom Vorübergehn der Stäbe
so müd geworden, daß er nichts mehr hält.
Ihm ist, als ob es tausend Stäbe gäbe
und hinter tausend Stäben keine Welt.

Der weiche Gang geschmeidig starker Schritte,
der sich im allerkleinsten Kreise dreht,
ist wie ein Tanz von Kraft um eine Mitte,
in der betäubt ein großer Wille steht.

Nur manchmal schiebt der Vorhang der Pupille
sich lautlos auf –. Dann geht ein Bild hinein,
geht durch der Glieder angespannte Stille –
und hört im Herzen auf zu sein.

- Rainer Maria Rilke (1903)

RÉSUMÉ

La contamination anthropogénique des régions côtières et estuaires a des conséquences pour la santé de l'écosystème. L'exposition des organismes de niveau trophique aux contaminants historiques a été corrélé à des défauts de reproduction, à l'apparition de cancer, et à la favorisation de maladies infectieuses qui peuvent conduire parfois à des évènements de mortalité de masse. Les principes actifs pharmaceutiques représentent une catégorie de contaminants d'intérêt croissant du fait de leur petite taille, de leur habileté à traverser les membranes, et de leur capacité à interagir avec les systèmes biologiques. L'étude de l'effet potentiel de leur combinaison entre eux ou à d'autres familles de composés en milieu aquatique en n'est qu'à ses premiers balbutiements.

Mon travail de thèse se décompose en trois études ayant pour but l'évaluation de l'effet que des composés pharmaceutiques, seuls ou en combinaison, peuvent avoir sur le système immunitaire adaptatif des phoques communs (*Phoca vitulina*). Un ensemble de biomarqueurs a été utilisé pour évaluer la toxicité aigüe des principes actifs pharmaceutiques au niveau cellulaire ainsi que plus finement au niveau de l'expression de certains gènes. L'objectif principal était d'établir des courbes dose-réponse, ainsi que de déterminer la toxicité (IC_{50}) pour les composés sélectionnés.

La première étude consistait à évaluer de l'immunotoxicité de composés pharmaceutiques sur une lignée cellulaire dérivée d'un lymphome à cellules B de phoque commun. Des biomarqueurs comme la prolifération lymphoblastique, le cycle cellulaire et l'apoptose, ainsi que la viabilité ont été utilisés pour mesurer le niveau de toxicité de composés pharmaceutiques, seuls ou en combinaison. La prolifération lymphoblastique ainsi que le cycle cellulaire ont été affectés lors de l'utilisation de certains composés. Par ailleurs, les tests (à l'exception de celui de viabilité) ont révélé des effets synergiques lors du traitement avec des combinaisons de composés.

La deuxième étude consistait à évaluer de l'immunotoxicité de composés pharmaceutiques sur des cultures primaires de lymphocytes T de chiots de phoques communs en liberté. Dans cette étude, la prolifération lymphocytaire a été inhibée alors que la viabilité est demeurée globalement inchangée lors de traitements avec les composés, seuls ou en combinaison. Les effets des combinaisons de composés n'ont pas été additifs. Les lymphocytes T étaient plus

sensibles aux composés individuels que la lignée cellulaire dérivée d'un lymphome à cellules B, mais moins sensibles aux combinaisons.

Lors de la troisième étude, nous voulu vérifier si le composé pharmaceutique le plus toxique lors des études précédentes (17α-ethinyl estradiol, EE_2), à des concentrations inférieures, était capable d'interférer avec l'expression de certains gènes, dans le but d'identifier plus précisément les mécanismes de toxicité. Nous avons observé une toxicité sur la lignée cellulaire dérivée d'un lymphome à cellules B associée a une augmentation importante de l'expression de l'ARNm de la protéine de choc thermique de 70 kDa uniquement pour les concentrations entrainant des effets sur la prolifération et le cycle cellulaire.

Dans son ensemble, ce travail a suggéré que le système immunitaire du phoque commun pouvait être affecté par l'exposition à des composés pharmaceutiques présents dans l'environnement, cependant à des concentrations supérieures à celles mesurées actuellement.

ABSTRACT

Anthropogenic contamination of coastal regions and estuaries has consequences for the ecosystem's health. Exposure to legacy contaminants in organisms of higher trophic levels has been linked to an impairment of reproduction, occurrence of cancer, and an increase in infectious diseases that even led to mass mortality events. Active pharmaceutical ingredients (APIs) are a contaminant class of emerging interest, since the molecules are rather small, penetrate membranes easily and were designed to interact with a biological system. Their potential effect in the cocktail of substances in the aquatic environment just begun to be discovered.

During the course of this dissertation work, three studies were implemented to elucidate the effect that pharmaceuticals and pharmaceutical mixtures can have on the adaptive immune system of harbor seals (*Phoca vitulina*). A battery of biomarkers was used to assess the acute toxicity of APIs on the cellular as well as the gene expression level. The main objective was to determine dose-response curves and IC_{50} values for the selected compound list.

In a first study, immunotoxicity of pharmaceuticals on a harbor seal B cell lymphoma cell line was assessed. Biomarkers like lymphocyte proliferation, cell cycle and apoptotic events as well as viability were used to determine the range of toxicity of individual pharmaceuticals and pharmaceutical mixtures. Both lymphocyte proliferation and the cell cycle were impacted by some of the individual compounds, and mixtures demonstrated synergistic effects in all assays, but the viability assay.

In a second study, the immunotoxic effect of pharmaceuticals on T lymphocytes of free-ranging harbor seal pups was determined. Lymphocyte proliferation was inhibited while viability remained mainly unaffected for all individual compounds and mixtures tested. Mixtures had non-additive mixed effects. T lymphocytes were more sensitive to individual compounds than the B cell lymphoma cell line, but less sensitive to binary mixtures of pharmaceuticals than the cell line.

In a third study, we wanted to assess if the most toxic pharmaceutical compound in our study (17α-ethinyl estradiol, EE_2) would present toxic effects at lower concentrations at the gene expression level compared to the cellular level that was tested in previous studies, and furthermore discover potential mechanisms of action of the toxic response. We observed a

toxicity towards the harbor seal B lymphoma cell line with a strong increase of the 70 kDa heat shock protein mRNA in those concentrations only that caused effects at the cellular level (i.e. lymphocyte proliferation and cell cycle changes).

Overall this work suggests that the immune system of harbor seals can be affected by an exposure to pharmaceutical compounds, however at higher concentrations than currently found in the environment.

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LIST OF ABREVIATIONS

A	**Ag**	Silver
	AhR	Aryl hydrocarbon receptor
	Al	Aluminum
	ANOVA	Analysis Of Variance
	APIs	Active pharmaceutical ingredients
	As	Arsenic
B	**ß2M**	beta-2-microglobulin
	ßACT	Beta-actin
	BaP	Benzo-alpha-pyrene
	BDE47	2,2',4,4'- tetrabromodiphenyl ether
	Be	Beryllium
C	**CAF**	Caffeine
	CAS No.	Chemical Abstracts Service Number
	CD#	Cluster of differentiation
	$CdCl_2$	Cadmium chloride
	cDNA	complementary DNA
	CO	Colorado
	CO_2	Carbon dioxide
	ConA	Concanavalin A
	COX-1/-2	Cyclooxygenase-1/-2
	CPM	Counts Per Minute
	Ct	Cycle threshold
	CZP	Carbamazepine
D	**DDT**	Dichlorodiphenyltrichloroethane
	DMSO	Dimethyl sulfoxide
E	**E_2**	17ß-estradiol
	EC_{50}	Half-maximal effective concentration
	EE_2	17α-ethinyl estradiol
	ERY	Erythromycin
	EtOH	Ethanol
F	**FBS**	Fetal Bovine Serum
	FSC	Forward Scatter

G	**G_0**	Gap 0/Resting phase
	G_1	Gap 1 phase
	G_2	Gap 2 phase
	GEM	Gemfibrozil
	GMP	Good Manufacturing Practice
H	**H_2O**	Water
	HBCD	Hexabromocyclododecane
	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
	Hg	Mercury
	HSP70	70 kDa Heat Shock Protein
I	**IBU**	Ibuprofen
	IC_{50}	Half-maximal inhibitory concentration
	IFNγ	Interferon-gamma
	Ig(G/M)	Immunoglobulin (G/M)
	IL-#	Interleukin-1/-2/-4/-6/-12/-13/-18
	IMS Health	Intercontinental Marketing Services – Health
L	**LC_{50}**	Half-maximal lethal concentration
	LOEC	Lowest Observed Effect Concentration
	$logK_{ow}$	decadic logarithm of the octanol-water partition coefficient
	LPS	Lipopolysaccharide
	LYN1	Lck/Yes novel tyrosine kinase
M	**M**	Mitosis phase
	MAPK3	Mitogen-activated protein kinase 3
	MAPKK3	Mitogen-activated protein kinase 3 kinase
	MeHg	Methylmercury
	MHC	Major Histocompatibility Complex
	mRNA	messenger RNA
N	**NADPH**	Nicotinamide adenine dinucleotide phosphate + hyrdrogen
	NAP	Naproxen
	NC	Negative control
	Ni	Nickel
	NK cell	Natural killer cell
	NSAIDs	Non-steroidal anti-inflammatory drugs
O	**OR**	Oregon

P	**PAHs**	Polycyclic aromatic hydrocarbons
	PAR	Paroxetine
	Pb	Lead
	PBDEs	Polybrominated diphenyl ethers
	PBMCs	Peripheral Blood Mononuclear Cells
	PBS	Phosphate-buffered saline
	PC	Positive control
	PCB	Polychlorinated Biphenyls
	Pen-Strept	Penicillin-Streptomycin
	PHA	Phytohaemagglutinin
	PI	Propidium iodide
	POPs	Persistent organic pollutants
	PPARs	Peroxisome Proliferator-Activated Receptors
	PPCPs	Pharmaceuticals and Personal Care Products
	PWM	Pokeweed mitogen
Q	**qPCR**	quantitative Polymerase Chain Reaction
R	**ROS**	Reactive Oxygen Species
	RPMI	Roswell Park Memorial Institute medium
	RT	Room Temperature
S	**S**	Synthesis phase
	SD	Standard Deviation
	Se	Selenium
	SEM	Standard Error or the Mean
	SLAM	Signaling lymphocytic activation molecule
	SMX	Sulfamethoxazole
	SSC	Side Scatter
	SSRIs	Selective Serotonin Reuptake Inhibitors
	STPs	Sewage Treatment Plants
T	**Ti**	Titanium
	TNFα	Tumor Necrosis Factor - alpha
	TGFß	Transforming Growth Factor - beta
	TMP	Trimethoprim
U	**U.S.A.**	United States of America
	UV	Ultraviolet

V	**V**	Vanadium
	V	Vehicle control
W	**WWTPs**	Waste water treatment plants
Y	**YWHAZ**	14-3-3 protein zeta/delta
Z	**Zn**	Zinc

VALORISATION DES TRAVAUX

Publications scientifiques

Accepté

C. Kleinert, M. Blanchet, F. Gagné, M. Fournier. Dose-response relationships in gene expression profiles in a harbor seal B lymphoma cell line exposed to 17α-ethinyl estradiol. Journal of Xenobiotics. (accepté le 28.04.2017)

C. Kleinert, E. Lacaze, M. Mounier, S. De Guise, M. Fournier. Immunotoxic effects of single and combined pharmaceuticals exposure on a harbor seal (*Phoca vitulina*) B lymphoma cell line. Marine Pollution Bulletin. DOI : 10.1016/j.marpolbul.2017.02.041 (accepté le 14.02.2017)

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En preparation

C. Kleinert, E. Lacaze, M. Fortier, M. Hammill, S. De Guise, M. Fournier. T Lymphocyte-Proliferative Responses of Harbor Seal (*Phoca vitulina*) PBMCs Exposed to Pharmaceuticals *in Vitro*. Marine Pollution Bulletin.

Communications orales

(Le nom souligné correspond à la personne ayant fait la presentation)

C. Kleinert, M. Fournier . L'impact des mélanges de produits pharmaceutiques sur les lymphocytes B de phoque. ÉcoBIM. Québec, Canada. 18.05. – 22.05.2015

C. Kleinert, M. Mounier, S. De Guise, M. Fournier . Impact of pharmaceuticals (single compounds and mixtures) on harbor seal B lymphocytes. SETAC North America. Vancouver, Canada. 09.11. – 13.11.2014

C. Kleinert, M. Mounier, S. De Guise, M. Fournier. L'impact des mélanges de produits pharmaceutiques sur les lymphocytes B de phoque. ÉcoBIM. Brest, France. 20.05. – 23.05.2014

C. Kleinert, M. Fortier, P. Brousseau, F. Gagné, S. De Guise, M. Fournier. 2013. Effets de diverses substances sur la compétence de cellules immunitaires de phoque suite à des expositions *in vitro.* ÉcoBIM. Montréal, Canada, 27.05. - 30.05.2013

Communications par affiches

(Le nom souligné correspond à la personne ayant fait la presentation)

C. Kleinert, M. Fournier. Dose-response relationships in gene expression profiles in a harbor seal cell line exposed to pharmaceuticals. 21st Biennial Conference on the Biology of Marine Mammals. San Francisco, U.S.A. 13.12. – 18.12.2015

C. Kleinert, E. Lacaze, M. Fortier, M. Fournier. L'impact des mélanges de produits pharmaceutiques sur les cellules immunitaires de phoque juvénile. Congrès Armand-Frappier. Orford, Canada. 12.11. – 14.11.2015 (premier prix « meilleure présentation par affiche »)

C. Kleinert, M. Fournier . L'impact des mélanges de produits pharmaceutiques sur les lymphocytes B de phoque. ÉcoBIM. Québec, Canada. 18.05. – 22.05.2015

C. Kleinert, M. Mounier, S. De Guise, M. Fournier. Impact of pharmaceuticals (single compounds and mixtures) on harbor seal B lymphocytes. SETAC North America. Vancouver, Canada. 09.11. – 13.11.2014

C. Kleinert, M. Mounier, M. Fortier, S. De Guise, M. Fournier. 2013. Effets des composés pharmaceutiques et de mélanges sur le système immunitaire de phoques communs (*Phoca vitulina*). Congrès Armand-Frappier. Orford, Canada. 14.11. – 16.11.2013

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C. Kleinert, M. Fortier, P. Brousseau, F. Gagné, S. De Guise, M. Fournier. Immunotoxic effects of pharmaceuticals in harbor seal (*Phoca vitulina*) lymphocytes in vitro. PRIMO 17. Faro, Portugal. 05.05. - 08.05.2013

Section 1.

General Introduction

1 GENERAL INTRODUCTION

Anthropogenic contamination of the environment has concerned the scientific community as well as the general public for several decades. Determining the concentrations of contaminants occurring in the environment and their toxic dose and mode of action has therefore been a priority for the field of ecotoxicology that emerged from these new research needs.

A large body of research has been conducted on legacy contaminants like persistent organic pollutants (POPs). The weight of evidence has led to the labeling of the initial "dirty dozen" consisting of pesticides, industrial chemicals and by-products during the 2001 Stockholm Convention. Since, the list has been expanded and the 180 parties of the convention have committed themselves to eliminate, restrict or reduce the unintentional releases of the chemicals on the list. More than a decade after the convention was signed, the accumulation in marine top-predators shows downward trends for some contaminants on the list (e.g. organochlorine pesticides, PBDE and HBCD flame retardants, butyltins) (Law, 2014). These are examples of successful risk assessment and management.

Active pharmaceutical ingredients (APIs) are compounds of emerging interest. Far less research has been conducted for these compound classes in comparison with POPs. Pharmaceuticals are of concern since the molecules are rather small, pass membranes and were designed to interact with proteins in organisms.

This study aims to assess the immunotoxic effects of exposure to selected pharmaceuticals on marine mammal leukocytes. Harbor seal leukocytes from blood samples of free-ranging animals as well as from the 11B7501 B cell lymphoma cell line were exposed to a range of APIs, and effects on the cellular as well as gene expression level were measured. We were able to determine the half-maximal inhibitory concentrations (IC_{50}) of all pharmaceuticals and mixtures thereof and compare sensitivity of different cell types. This was the first study assessing the effects of pharmaceuticals on the immune system of a marine mammal.

HYPOTHESIS AND OBJECTIVES

Hypothesis

- Active pharmaceutical ingredients in municipal effluents have the potential to disturb the function of the immune system in harbor seals.
- Chronic exposure to pharmaceuticals could disturb the organism's resistance towards pathogens.

Main Objectives

- Evaluate two *in vitro* models for the study of immunotoxicity.
- Elucidate the toxic mechanisms of action of pharmaceuticals in seals.
    - On the cellular level (through immune responses)
    - On the biochemical level (through analysis of gene expression)

Specific Objectives

- **Chapter 1:** Immunotoxicity of pharmaceuticals on a B cell lymphoma cell line
    - Evaluation of pharmaceutical toxicity and mixture toxicity on B lymphocytes with a harbor seal 11B7501 B cell lymphoma cell line
- **Chapter 2:** Immunotoxicity of pharmaceuticals on T lymphocytes
    - Evaluation of pharmaceutical toxicity and mixture toxicity on T lymphocytes with harbor seal peripheral blood mononuclear cells (PBMCs)
    - Comparison of compounds, mixtures, sexes, and PBMCs vs. 11B7501 cell line
- **Chapter 3:** Immunotoxicity of pharmaceuticals on gene expression
    - Evaluation of EE_2 toxicity on harbor seal B cell lymphoma cell line gene expression
    - Develop harbor seal primers for selected immune-specific mRNA sequences
    - Compare sensitivity of RT-qPCR with responses obtained from Chapter 1

Section 2.

Literature Review

2 LITERATURE REVIEW

2.1 Pharmaceuticals in the Canadian environment

The presence of pharmaceutically active compounds (or active pharmaceutical ingredients, APIs) in the environment led to emerging concern in the past decades. APIs are complex molecules with a wide range of physiochemical and biological properties and functionalities. They are small molecules (200-1000 Dalton) that are developed with broad or specific biological activity (Kümmerer, 2008).

The wide range of chemical properties of APIs makes an assessment of their environmental effects complex. APIs can have basic or acidic functionalities, and molecules can be neutral, cationic, anionic, or zwitterionic (having both positive and negative electrical charge). For some molecules, like the antibiotic ceftazidime, the $\log K_{ow}$ varies with pH (Kümmerer, 2008). Also, potential effects of metabolites of the parent compound and post-excretion transformation products have to be taken into account, since these could significantly change the physiochemical properties, in comparison with the parent compound, and bioavailability to non-target organisms (Kümmerer, 2008).

A further challenge in the field of eco-pharmacology is the assessment of differences in the occurrence of APIs, their fate, and their effects on target and non-target organisms. Being aware of the fate and (eco-)toxic effects of these compound classes is fundamental for later risk-assessment and management.

2.1.1 Pharmaceutical Sales and Consumption

Non-prescription pain relievers (analgesics) are some of the most commonly sold pharmaceuticals in Canada. According to IMS Health unit sales data from 2001, acetaminophen, acetylsalicylic acid, ibuprofen and naproxen sales are ranging from 10,000 to 1,000,000 kg/year (Metcalfe *et al.*, 2004). Sales of the hormone 17α-ethinyl estradiol (EE_2), the active compound of some contraceptive pills, are below 100 kg/year. Nevertheless, EE_2 is still present in the aquatic environment in the ng/L range in Canada (Metcalfe *et al.*, 2004) and other countries (Aris *et al.*, 2014).

Application and consumption of pharmaceutical compounds vary significantly between countries (Goossens *et al.*, 2007, Goossens *et al.*, 2005, Verbrugh *et al.*, 2003). The EE_2 contraceptive pill is taken by 16 % of women in the reproductive age in North America compared to only 0.4 % in Japan (Kümmerer, 2008). And, whereas Germany banned the use of the antibiotic streptomycin in fruit agriculture due to research linking those compounds to the occurrence of highly streptomycin-resistant pathogenic bacteria, the U.S.A. continue to use it (Boxall *et al.*, 2004, Boxall *et al.*, 2003, Sarmah *et al.*, 2006).

It is very difficult to estimate what the actual input into the Canadian and global sewage treatment systems is. For estimations, one has to not only consider sales and consumption, but also prescription rates, therapeutic doses and different rates of metabolism after consumption (Metcalfe *et al.*, 2004). Therefore, it is fundamental to determine primary sources of contamination and measure concentrations of priority compounds in the aquatic and terrestrial environment.

2.1.2 Pharmaceuticals in Water and Sediment

2.1.2.1 Sources of contamination

Due to the production, application and consumption of pharmaceuticals by humans, APIs, and their metabolites are present in urban wastewater (Metcalfe *et al.*, 2004). Whereas European and North American manufacturers of the pharmaceutical industry have to abide by Good Manufacturing Practice (GMP) regulations, and therefore emit negligible amounts into urban wastewater, pharmaceutical concentrations in effluents in Asian countries can be up to several mg/L (Larsson, 2008, Larsson *et al.*, 2007, Li *et al.*, 2008). Point sources such as hospitals are generally seen as minor sources for pharmaceuticals in the environment (Heinzmann *et al.*, 2006, Kümmerer *et al.*, 2008).

Private households, however, are a significant source of these contaminants, either since medication is consumed and excreted as its original or metabolized form, or because people dispose of their outdated and unwanted medications via household drains (toilet or sink), or trash them (Figure 1). Trash containing pharmaceuticals that is not incinerated but deposited in landfills can leach these compounds into landfill effluent (Kümmerer, 2008).

Next to pharmaceuticals for human consumption, veterinary drugs are an important factor in environmental contamination by pharmaceuticals (Figure 1). Parent compounds and their metabolites are excreted by live stock in manure which is used (along with sewage sludge) to fertilize fields (Kümmerer, 2008). Thus, APIs are introduced into the soil and surrounding surface waters as run-off (Metcalfe *et al.*, 2004).

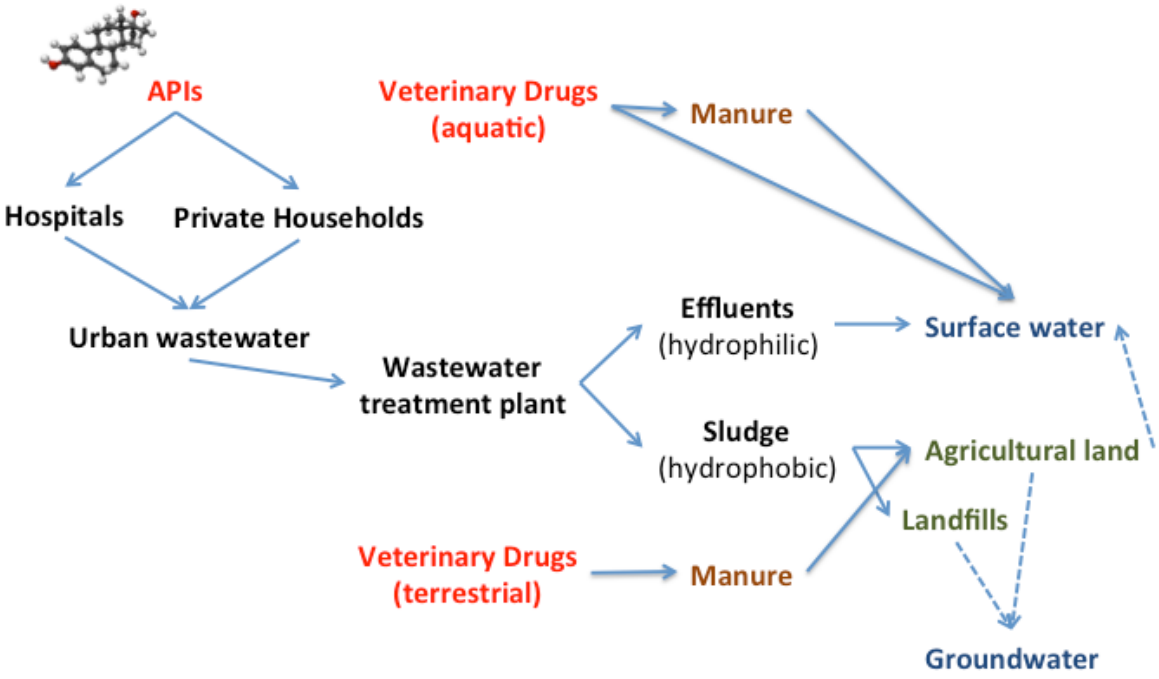


Figure 1. Sources of contamination with active pharmaceutical ingredients (APIs) for human and veterinary use and pathways (adapted from (Kümmerer, 2008, Metcalfe *et al.*, 2004))

In this work, we focus on 10 pharmaceutical compounds that cover a wide range of pharmaceutical classes including non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, a lipid regulator, neuroactive compounds (antidepressants, antiepileptic drugs, stimulants), and a synthetic hormone (Table 1). The compounds selected have previously shown immunotoxic effects in invertebrate studies (Table 8).

The majority of the compounds chosen have been detected in the St. Lawrence River (details on concentrations see Table 2) (Gagné *et al.*, 2006a). Furthermore, the compound list has been complemented with an anti-depressant (paroxetine), a synthetic hormone (17α-ethinyl estradiol, EE_2) and an additional antibiotic (erythromycin).

Table 1. Physico-chemical properties of the selected pharmaceutical compounds

Compounds	Brief	CAS No.	Molecular formula	Molecular weight	$logK_{ow}$	pKa
Caffeine	CAF	58-08-2	$C_8H_{10}N_4O_2$	194.20	-0.01	14.0 (25 ˚C)
Carbamazepine	CZP	298-46-4	$C_{15}H_{12}N_2O$	236.27	2.47	13.9
Erythromycin	ERY	114-07-8	$C_{37}H_{69}NO_{14}$	751.94	3.06	8.9 (25 ˚C)
17α-ethinyl estradiol	EE_2	57-63-6	$C_{20}H_{24}O_2$	296.40	3.67	10.2
Gemfibrozil	GEM	25812-30-0	$C_{15}H_{22}O_3$	250.34	4.77	4.75
Ibuprofen	IBU	15687-27-1	$C_{13}H_{18}O_2$	206.28	3.97	4.9
Naproxen	NAP	22204-53-1	$C_{14}H_{14}O_3$	230.26	3.50	4.2
Paroxetine	PAR	61869-08-7	$C_{19}H_{20}FNO_3$	329.37	3.60	9.6
Sulfamethoxazole	SMX	723-46-6	$C_{10}H_{10}N_3O_3S$	253.28	0.89	5.6
Trimethoprim	TMP	738-70-5	$C_{14}H_{18}N_4O_3$	290.32	1.33	7.1 (20 ˚C)

An anti-depressant was included, since these compounds are one of the most commonly prescribed drugs in North America (NCHS, 2011), and therefore omnipresent in the limnic system (Daughton *et al.*, 1999). Selective serotonin reuptake inhibitors (SSRIs) have been implicated with decreased predatory avoidance behavior in fathead minnow larvae (*Pimephales promelas*) or reduced aggression in male Siamese fighting fish (*Betta splendens*) (Dzieweczynski *et al.*, 2012, Painter *et al.*, 2009).

Furthermore, the synthetic estrogen EE_2 was added, since it is a compound that is omnipresent at ng/L concentrations in almost all western river systems (Aris *et al.*, 2014). Like all hormones, synthetic estrogens are highly effective at low concentrations, and have been therefore implicated in alterations of sex determination, delayed sexual maturity, and decrease in secondary sexual characteristics in fish (Aris *et al.*, 2014).

Erythromycin has been selected in addition to the antibiotics sulfamethoxazole and trimethoprim. These three antibiotics have been determined as interesting molecules to monitor for immunotoxic effects in a study on the freshwater mussel *Elliptio complanata* (Gust *et al.*, 2012).

The effects of a mixture of all antibiotics in the study were principally explained by these three compounds (Gust *et al.*, 2012). Furthermore, erythromycin has been recently added to the EPA watch list of potentially harmful contaminants.

2.1.2.2 Concentrations in Effluents

Sewage treatment plants remove a multitude of anthropogenic contaminants from urban and municipal wastewater. Contaminants are transformed and waste water is purified through physical, chemical, and biological processes until its release into the environment. Primary treatment usually consists of sewage being introduced into sedimentation basins where large particles, soluble substances and lipids are separated. Secondary treatments are designed to remove a majority of the organic content and include processes like activated sludge, aerated lagoons, aerobic granulation, constructed wetlands, membrane bioreactors, rotating biological contactors, sequencing batch reactors, and trickling filter (Tchobanoglous *et al.*, 1991). Tertiary treatments remove further contaminants from the effluent to increase its quality, and one or more treatments might be applied before effluent is released into the receiving aquatic environment. Processes include sand or activated carbon filtration, lagoons or ponds, nitrogen and phosphorus removal, and disinfection with UV light, ozone, chlorine, or sodium hypochlorite (Tchobanoglous *et al.*, 1991).

Due to the physicochemical properties of different molecules, treatment cannot be adapted for every single compound present in the wastewater. For instance, basic pharmaceuticals and zwitterions have been shown to effectively adsorb to sewage sludge (Golet *et al.*, 2002). Acidic pharmaceuticals, such as NSAIDs (e.g. ibuprofen, naproxen), remain soluble in the water column and rely on biodegradation to be eliminated (Buser *et al.*, 1998, Kümmerer *et al.*, 1997, Ternes *et al.*, 2004).

The removal rate of some pharmaceuticals can be quite low, depending on the treatment technology and performance of the wastewater treatment plant (WWTP), hydraulic retention time and season (Fent, 2008). Carbamazepine is one of the drugs resisting treatment to a great extend with removal rates ranging between 7-8 % (Clara *et al.*, 2004, Heberer, 2002, Ternes, 1998). In a Montréal WWTP removal was merely 6 % after primary treatment (Gagnon *et al.*, 2012). Another Canadian study reported a 4.9 % removal efficiency after primary treatment and

an average of 5.8 % removal efficiency after secondary treatment (Lajeunesse *et al.*, 2012). On the other hand, drugs like salicylic acid and anti-inflammatory compounds are oftentimes eliminated with up to 99 % efficiency (Heberer, 2002, Ternes, 1998, Ternes *et al.*, 1999a). Removal rates for ibuprofen (20 %) and naproxen (33 %) in the previously mentioned Montréal WWTP were however significantly lower (Gagnon *et al.*, 2012).

A Canadian study by Rogers *et al*., 1986 was one of the first reports to demonstrate the release of APIs from municipal effluent into the environment (Rogers *et al.*, 1986). When effects of pharmaceuticals in the environment have become more evident in the mid-1990s, a lot of efforts have been made globally to determine concentrations of APIs in effluents (Kümmerer, 2008).

Few studies sampled Canadian WWTPs until the beginning of the 21st century. A 2004 review of the Canadian situation (Metcalfe *et al.*, 2004) cited only four of their own studies that sampled Canadian WWTP effluents between 1999-2002 (Hua *et al.*, 2006, Metcalfe *et al.*, 2003a, Metcalfe *et al.*, 2003b, Miao *et al.*, 2002). The most frequently detected drug was anti-epileptic carbamazepine and the most frequently detected anti-inflammatory drug was ibuprofen. Both anti-inflammatory drugs ibuprofen and naproxen were often present at µg/L concentrations in effluents (Metcalfe *et al.*, 2004).

Lishman *et al*. furthered the Canadian database by detecting 6/18 measured APIs in influent and effluent of 12 municipal WWTPs discharging into the Thames River in southwestern Ontario (Lishman *et al.*, 2006). In the same year, Gagné *et al*., 2006a detected 12/40 pharmaceuticals in effluents discharging into the St. Lawrence River around Montreal in Quebec (Gagné *et al.*, 2006a).

Table 2 summarizes a selection of studies conducted in Canada that assessed the amount of pharmaceuticals in wastewater effluents. A special focus was drawn to studies conducted in the province of Quebec. The pharmaceuticals listed below are solely a small percentage of the more than 200 compounds found in Canadian and global effluents.

Table 2. Concentrations of selected drugs in treated effluent samples collected from Canadian WWTPs with focus on studies conducted in the province Québec, as reported by (Metcalfe *et al.*, 2003a, Metcalfe *et al.*, 2003b, Miao *et al.*, 2002), (Metcalfe *et al.*, 2003b) Hua *et al.* 2004a,b, Miao *et al.* 2004)[1], (Gagné *et al.*, 2006a) (mean not median)[2], (Lajeunesse *et al.*, 2007)[3], (Lajeunesse *et al.*, 2012)[4], (Ternes *et al.*, 1999b)[5], (Lishman *et al.*, 2006)[6], (Lajeunesse *et al.*, 2008)[7] and (Segura *et al.*, 2007)[8]

Analyte	Median (µg/L)	Max (µg/L)	# samples (total)	# samples (ND)	Sampling Location	Sampling Year
Caffeine	0.022[1]	0.677	7	0	Pan Canadian	1999-2002
	11.160[2]	22.000	3		Montréal, QC	<2005
Carbamazepine	0.107[1]	2.3	26	0	Pan Canadian	1999-2002
	0.085[2]	0.137	3		Montréal, QC	<2005
	0.656[3]		3		Montréal, QC	2005
	0.100[3]		2		Montréal, QC	2005
	0.091[3]		3		Montréal, QC	2006
	0.923[4]		6		Pan Canadian	2009
	0.914[4]		6		Pan Canadian	2010
	0.397[4]		6		Pan Canadian	2009
	0.404[4]		6		Pan Canadian	2009
	0.961[4]		6		Pan Canadian	2009
	0.681[4]		6		Pan Canadian	2009
	2.956[4]		6		Pan Canadian	2009
Erythromycin-H_2O	0.080[1]	0.838	8	0	Pan Canadian	2003
17α-ethinyl estradiol	0.009[5]	0.042	10	1	ON	1998
Gemfibrozil	0.043[1]	2.174	26	17	Pan Canadian	1999-2002
	0.071[2]	0.084	3		Montréal, QC	<2005
	0.255[6]	0.436		22	ON	2002
Ibuprofen	1.885[1]	26.6	26	7	Pan Canadian	1999-2002
	0.786[2]	1.191	3		Montréal, QC	<2005
	0.858[3]		3		Montréal, QC	2005
	1.060[3]		2		Montréal, QC	2005
	0.609[3]		3		Montréal, QC	2006
	0.353[6]	0.773		23	ON	2002

Naproxen	0.168[1]	0.855	26	15	Pan Canadian	1999-2002
	0.271[2]	0.325	3		Montréal, QC	<2005
	2.579[3]		3		Montréal, QC	2005
	0.382[3]		2		Montréal, QC	2005
	0.217[3]		3		Montréal, QC	2006
	0.351[6]	1.189		18	ON	2002
Paroxetine	0.012[4]		6		Pan Canadian	2009
	0.005[4]		6		Pan Canadian	2010
	0.0063[4]		6		Pan Canadian	2009
	0.0037[4]		6		Pan Canadian	2009
	0.0013[4]		6		Pan Canadian	2009
	0.006[4]		6		Pan Canadian	2009
	0.004[4]		6		Pan Canadian	2009
	0.0043[7]		3		Montréal, QC	2007
	0.0052[7]		3		Montréal, QC	2007
Sulfamethoxazole	0.243[1]	0.871	8	0	Pan Canadian	2003
	0.049[2]	0.099	3		Montréal, QC	<2005
	0.100[8]				Montréal, QC	2006
Trimethoprim	0.071[1]	0.194	7	0	Pan Canadian	1999-2002
	0.065[2]	0.070	3		Montréal, QC	<2005
	0.100[8]				Montréal, QC	2006

Abbreviations: ND = not detected

2.1.2.3 Concentrations in Surface water

Wastewater treatment plant effluents are introduced into the aquatic environment by feeding them into the adjacent rivers. This way, anthropogenic xenobiotics, particularly pharmaceuticals, reach the surface waters. WWTP effluents represent a major point source of pharmaceuticals in riverine environment (Grover *et al.*, 2011, Zhou *et al.*, 2009). Pharmaceutical concentrations found in effluents will be diluted in surface water. For example, carbamazepine concentrations varied in a British river upstream (53 ng/L), downstream (180 ng/L) and in proximity to an effluent output (265 ng/L) (Zhou *et al.*, 2014).

Next to distance from the nearest WWTP, season and weather conditions can strongly impact the concentrations of pharmaceuticals in surface waters. Zhou *et al*., 2014 found pharmaceutical concentrations to be the highest in the beginning of summer, where rainfall is low causing limited dilution and where consumption of pharmaceuticals might be higher.

All ten of the selected pharmaceutical compounds in our study have been detected in surface water in North America and Europe (Table 2). Caffeine was the compound with the highest maximal value of all ten selected pharmaceuticals at 6 µg/L in a U.S. study, (Table 3; (Kolpin *et al.*, 2002)). The antibiotics erythromycin and sulfamethoxazole have been other compounds with maximal values in the µg/L range in U.S. streams (1.7 and 1.9 µg/L, respectively) (Table 3; (Kolpin *et al.*, 2002)).

Sea water has been rarely analyzed for its pharmaceutical content (Weigel *et al.*, 2001, Weigel *et al.*, 2002). The majority of studies focused on the limnic system, especially rivers. Due to the dilution factor, concentrations in the open sea are typically lower (pg/L to lower ng/L range) in comparison with rivers, and sampling methods have to be specifically adapted to handle higher volumes and flow rates (Weigel *et al.*, 2001). Caffeine has been found in the North Sea at 6.45 ng/L (Weigel *et al.*, 2002), while values in North American Rivers had median concentrations ranging from 17 to 81 ng/L (Kolpin *et al.*, 2002, Metcalfe *et al.*, 2003a)). Weigel *et al*. 2001 were the first group to report caffeine and carbamazepine in sea water. Although scarce, obtaining sufficient data on the occurrence and distribution of pharmaceuticals in the marine environment is important, since (i) the presence of pharmaceuticals in sea water indicates a certain persistence of these compounds, and since (ii) the physico-chemical conditions are significantly different in the sea water, potentially leading to different behavior (e.g. stability) of these compounds (Weigel *et al.*, 2002).

The St. Lawrence River Estuary is characterized by the following physico-chemical parameters (averages given; deviations occur with depth and season): water temperature of ~ 4 ˚C; salinity of ~ 34; pH of 7.6 – 7.7 in the deep waters; dissolved organic carbon (DOC) of 2.5 – 5 mg C L^{-1} (Galbraith *et al.*, 2016, Gilbert *et al.*, 2005). The flow of xenobiotics is relatively high and contaminants can be retained temporarily (area of maximum turbidity in the upper estuary) or permanently (upper basin of the Saguenay River Fjord) (Savaria *et al.*, 2008). It is important to take these characteristics into account when assessing fate and bioavailability of xenobiotics.

Table 3. Concentrations of selected drugs in surface water samples collected in Canada with focus on studies conducted in the province Québec, as reported by Metcalfe et al. (2003a,b)[1], Miao et al. (2002)[1], Miao and Metcalfe (2003a-c)[1], Hua et al. (2004a,b)[1], Schultz et al. (2010)[2], Vazquez-Roig et al. (2012)[3], Zhou et al. (2014)[4], Carlson et al. (2013)[5,] Huang et al. (2001)[11], Koplin et al. (2012)[12], Belfroid et al. (1999)[6], Fawell et al. (2001)[10], Xiao et al. (2001)[13], Koplin et al. (2002)[12], Metcalfe et al. (2003b)[14], Weigel et al. (2002)[15], Boyd et al. (2001)[7], Boyd et al. (2003)[8], Buser et al. (1999)[9], Fawell et al. (2001)[10], Huang et al. (2001)[11], Koplin et al. (2002)[12], Xiao et al. (2001)[13], Metcalfe et al. (2003b)[14], Weigel et al. (2002)[15]. Weigel et al. (2001)[17]

Analyte	Median (µg/L)	Max (µg/L)	# samples (total)	# samples (ND)	Sampling Location	Sampling Year
Caffeine	0.017[1]	0.046	5	0	Pan Canadian	1999-2002
	0.081[12]	6			Pan USA	2002
	0.00645[15]	0.0161	14	0	North Sea	1998
	0.002[17]	0.002	1	0	North Sea	1998
Carbamazepine	0.020[1]	0.650	46	22	Pan Canadian	1999-2002
	0.0188[5]	0.0256	POCIS	1	DHC, MB	2010
	0.185[14]	0.650			Detroit River	2003
	0.120[14]	0.31			HH, ON	2003
	0.002[17]	0.002	1	0	North Sea	1998
	0.180[14]	0.265			Medway, UK	2010
	0.0055[3]	0.0388	26		Pego, Spain	2009
Erythromycin-H_2O	0.020[1]	0.034	4	1	Pan Canadian	2002
	<0.003[5]	<0.003	POCIS	3	DHC, MB	2010
	1,0[12]	1.7			Pan USA	2002
17α-ethinyl estradiol	<0.0045[5]	<0.0045	POCIS	2	DHC, MB	2010
		0.00007[11]			CA, USA	2001
	0.073[12]	0.831			Pan USA	2002
		0.0043[6]			Netherlands	1999
	0.00015[10]				UK	2001
	0.0001[13]				UK	2001
Gemfibrozil	0.012[1]	0.112	48	34	Pan Canadian	1999-2002
	0.0302	0.0466	POCIS	0	DHC, MB	2010
	0.0025[14]				Canada (EST)	2003
	0.012[14]	0.067			HH, ON	2003
	0.066[14]	0.112			Detroit River	2003
	0.048[12]	0.79			Pan USA	2002

Ibuprofen	0.064[1]	0.790	48	39	Pan Canadian	1999-2002
	0.025[14]				Canada (EST)	2003
	0.141[14]	0.79			Detroit River	2003
	0.2[12]	1			Pan USA	2002
	0.00175[8]				Pan USA	2003
	ND (<0.0002)[9]		2	2	North Sea	1999
	0.0006[15]		1		EE, Germany	1998
	0.0163[3]	0.059	74		Pego, Spain	2009
Naproxen	0.094[1]	0.551	48	34	Pan Canadian	1999-2002
	0.0021[5]	0.0024	POCIS	1	DHC, MB	2010
	0.0025[14]				Canada (EST)	2003
	0.270[14]	0.551			Detroit River	2003
	0.094[14]	0.139			HH, ON	2003
	<0.005[7]	0.02			USA	2001
	0.037[8]	0.107			USA	2003
Paroxetine	0.003[2]	0.004	12	3	BC, CO	2005
	0.0012[2]	0.0039	8	4	BC, CO	2006
Sulfamethoxazole	0.008[1]	0.099	4	0	Pan Canadian	2002
	0.020[5]	0.0484	POCIS	1	DHC, MB	2010
	0.150[12]	1.9			Pan USA	2002
	0.0016[3]	0.0156	32		Pego, Spain	2009
	0.005[4]	0.010			Medway, UK	2010
Trimethoprim	0.043[1]	0.134	5	2	Pan Canadian	1999-2002
	0.00264[5]	0.00674	POCIS	3	DHC, MB	2010
	0.150[12]	0.71			Pan USA	2002
	0.0001[3]	0.003	3		Pego, Spain	2009

Abbreviations: BC = Boulder Creek; CO = Colorado; DHC = Dead Horse Creek; EE = Elbe Estuary; EST = Eastern; HH = Hamilton Harbor; MB = Manitoba; Medway = River Medway; ND = not detected; ON = Ontario; POCIS = Polar Organic Chemical Integrative Samplers; UK = United Kingdom; USA = United States of America.

2.1.2.4 Concentrations in Sediment

The sediment is considered to be the most important contaminant reservoir in the St. Lawrence River Estuary (Savaria *et al.*, 2008). Furthermore, sediments provide an understanding of the long-term occurrence of xenobiotics in contrast to water samples (Antonić *et al.*, 2007). Nonetheless, there are no studies that have qualitatively or quantitatively assessed the occurrence of pharmaceuticals and personal care products (PPCPs) in the estuary's sediments. To our knowledge, there are only two studies that have assessed the occurrence of PPCPs in North American river sediments to date (Table 5; (Nilsen *et al.*, 2007, Schultz *et al.*, 2010)). The present section will therefore include studies from European countries as well, due to the similar consumption of pharmaceuticals and similar regulations for wastewater treatment.

The adsorption of pharmaceuticals from the water column to the river sediment does not depend solely on the hydrophobicity of the compound. Ionic interactions, pH of the water and molecular weight of the compound are critical variables, as well (Vazquez-Roig *et al.*, 2012, Zhou *et al.*, 2014).

Nilsen *et al.* 2007 analyzed pharmaceuticals in the sediments of the Columbia River, OR. Unfortunately, exact values for each pharmaceutical could not be extracted from the publication, since results were displayed in a graph rather than a table. It was, however stated that caffeine, carbamazepine and trimethoprim were in the range of 0.004 to >1.5 µg/g OC (organic carbon).

Paroxetine concentrations in sediments of the Boulder Creek, CO surged with increasing distance downstream of the WWTP (Table 4; (Schultz *et al.*, 2010)).

Table 4. Surface water and sediment concentrations of paroxetine in Boulder Creek, CO, U.S.A. sampled in 2005. Paroxetin concentrations diminish in surface water downstream the WWTP and increase in the sediments (Schultz *et al.*, 2010).

Concentrations	**Upstream**	**WWTP outfall**	**Downstream**	
(ng/L)	(- 0.1 km)	(0 km)	(+ 3.7 km)	(+ 8.1 km)
Surface water	ND	4.0	3.0	2.0
Sediment	ND	1.1	2.8	3.2

Abbreviations: ND = not detected; WWTP = wastewater treatment plant

Surface water PAR concentrations, however, were highest at the WWTP outfall. The spatial pattern of stream and sediments are therefore significantly different and show opposite trends (Table 4). The same trends were found for carbamazepine and sulfamethoxazole in a British study (Zhou *et al.*, 2014). These findings indicate that there can be a time lag between discharge and accumulation in sediment, likely related to the strength of the water flow (Zhou *et al.*, 2014).

Table 5. Concentrations of selected drugs in sediment samples collected in western countries, as reported by (Nilsen *et al.*, 2007)[1], (Schultz *et al.*, 2010)[2], (Vazquez-Roig *et al.*, 2012)[3]and (Zhou *et al.*, 2014)[4].

Analyte	Median (ng/g)	Max (ng/g)	# samples (total)	# samples (ND)	Sampling Location	Sampling Year
Carbamazepine	0.9[3]	1.7	100		Pego, Spain	2009
	~15[4]	46.5		0	Medway, UK	2010
Erythromycin	ND[1]				CR, OR	2007
Ibuprofen	ND[3]		0		Pego, Spain	2009
Paroxetine	2.16[2]	3.43	8	2	BC, CO	2005
Sulfamethoxazole	ND[1]				CR, OR	2007
	0.1[3]	1.1	13		Pego, Spain	2009
	1.1[4]	2.5			Medway, UK	2010
Trimethoprim	0.2[3]	1.6	20		Pego, Spain	2009

Abbreviations: BC = Boulder Creek; CO = Colorado; CR = Columbia River; ND = not detected; OR = Oregon; UK = United Kingdom.

2.1.3 Pharmaceuticals in Organisms

Due to the constant reintroduction of pharmaceuticals from WWTPs, pharmaceuticals are considered pseudo-persistent and have been detected in aquatic organisms. Table 6 summarizes examples of accumulation of selected pharmaceuticals in fish. To date, accumulation has been studied in individuals exposed in the laboratory as well as in field caught fish.

The accumulation in fish is stated here, since there are no studies to date on accumulation of the selected pharmaceuticals in harbor seals or other marine mammals. Furthermore, fish are chosen here over studies in mice or other traditional laboratory animals, since they are also aquatic animals, vertebrates and most importantly the prey of harbor seals. We hypothesize that the fish diet is the main route of exposure to pharmaceuticals for seals.

Most of the selected compounds in our study have been detected in fish tissue previously (Table 6). Concentrations in tissues were in the ng/g range and concentrations in plasma were in the µg/L range. For carbamazepine, a field study showed that liver accumulated this lipophilic compound more readily than muscle tissue. This observation was confirmed by Liu *et al*., 2015 who found the accumulation of lipophilic pharmaceuticals to be in the order of liver > brain > gill > muscle in free-ranging fish. SSRIs were also found in trout tissue in the order of liver > brain > muscle (Lajeunesse *et al.*, 2011).

Moreover, it has been shown that the pharmaceutical profile observed in fish tissue deviates from the profile in stream water and sediment, indicating a selective uptake in fish (Schultz *et al.*, 2010).

Table 6. Concentrations of selected drugs in fish tissue, as reported by (Wang *et al.*, 2013)[3], (Moore *et al.*, 2008)[4], (Ramirez *et al.*, 2009)[9], (Fick *et al.*, 2010a)[10], (Kim *et al.*, 2009)[11], (Li *et al.*, 2011)[12], (van den Brandhof *et al.*, 2010)[13], (Gelsleichter, 2009a)[17], (Larsson *et al.*, 1999)[18], (Schweinfurth *et al.*, 1996)[19], (Brown *et al.*, 2007)[21], (Henriques *et al.*, 2016)[22], (Knoll/BASF, 1995)[24], (Pounds *et al.*, 2008)[25], (Rodriguez *et al.*, 1992)[27], (Zhang *et al.*, 2010)[30], (Metcalfe *et al.*, 2010)[31] and (FDA-CDER, 1996)[32].

Analyte	Concentration in field caught fish [ng/g]	Concentration in experientally exposed fish (compartment/ exposure) [tissues ng/g; plasma µg/L]	Acute toxcicity in fish [mg/L]
Caffeine		17 (tissue / reclaimed water for 7 d)[3]	80-120 (48 h LC50 / *P. promelas*)[4]
Carbamazepine	<LOD-3.1 (fillet)[9] <LOD-8 (liver)[9]	1.8 (tissue / reclaimed water for 7 d)[3] 0.3-1.0 (plasma / UTSE for 14 d)[10]	35.4 (48 h LC50 / *O. latipes*)[11] 45.87 (96 h LC50 / *O. latipes*)[11] 19.9 (96 h LC50 / *O. mykiss*)[12] 86.5 (72 h EC50 / *O. mykiss*)[13]
Erythromycin-H_2O			>100 (96 h LC50 / *O. latipes*)[11]
17α-ethinyl estradiol	2.32 ±1.45 µg/L (plasma)[17]	380 (bile / 50 µg/L EE_2 for 46 h)[18]	1.6 (96 h EC50 / *O. mykiss*)[19]
Gemfibrozil	27-90 (liver)[9]	210-400 (plasma / UTSE for 16 d)[21]	11.01 (96 h LC50 / *D. rerio*)[22]
Ibuprofen		10 (tissue / reclaimed water for 7 d)[3] 5.5-102 (plasma / UTSE for 14 d)[10] 62-84 (plasma / UTSE for 16 d)[21]	>300 (NEL / *C. vaiegatus*)[24] 173 (96 h LC50 / *L. macrochirus*)[24] >100 (96 h LC50 / *O. latipes*)[25]
Naproxen		33-46 (plasma / UTSE for 14 d)[10] 9-14 (plasma / UTSE for 16 d)[21]	560 (96 h LC50 / *L. macrochirus*)[27] 690 (96 h LC50 / *O. mykiss*)[27]
Paroxetine	0.55 µg/L (plasma)[17] 0.48-0.58 (tissue)[31]	0.35 ± 0.1 (liver / primary-treated effluent for 3 months)[30] 0.19 ± 0.11 (brain)[30]	2 (LC50 / *L. macrochirus*)[32]
Sulfamethoxazole			>750 (48 h LC50 / *O. latipes*)[11] >562 (96 h LC50 / *O. latipes*)[11]
Trimethoprim			>100 (48 h LC50 / *O. latipes*)[11] >100 (96 h LC50 / *O. latipes*)[11]

Abbreviations: UTSE = Undiluted treated sewage effluent; NEL = no effect level; LC_{50} = half-maximal lethal concentration.

2.1.3.1 Toxicological impacts of pharmaceuticals in fish species

Since pharmaceuticals have been detected in fish tissue and since these molecules are designed to be biologically active (Weigel *et al.*, 2002), it is necessary to evaluate the potential interactions and toxic effects with this organism. In toxicology, EC_{50} (half maximal effective concentration) and LC_{50} (half maximal lethal concentration) values are quantified to evaluate the toxicity in acute, short-term exposure. Table 6 summarizes a selection of EC_{50} and LC_{50} values of selected pharmaceuticals in fish. These values are frequently used in risk assessment to calculate predicted no effect concentrations (PNECs) (Kim *et al.*, 2007, Lopez-Serna *et al.*, 2012, Vazquez-Roig *et al.*, 2011).

Long-term exposure data based on more specific endpoints as biomarkers at low concentrations are preferred to short-term data, but are largely lacking (Vazquez-Roig *et al.*, 2012). Previously, sublethal concentrations of pharmaceuticals have been documented to impair an organism's ability to function normally in its ecosystem (Gerhardt *et al.*, 2002). Therefore, it is crucial to evaluate the modes of action of sublethal toxic effects to extrapolate and potentially explain the effects in long-term exposure at lower concentrations. The following sections will summarize modes of toxic action in fish of different pharmaceutical classes.

Analgesics and Non-steroidal Anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat inflammation, pain, fever and in some cases for long-term treatment of rheumatic patients (Fent *et al.*, 2006). Most inhibit reversibly or irreversibly cyclooxygenase (COX-1 and/or COX-2) activity, leading to a reduced endogenous prostaglandin synthesis (Vane *et al.*, 1998). Prostaglandins have a multitude of hormone-like biological functions ranging from vasoconstriction in the kidney to the regulation of inflammation (Smith, 1971, Vane, 1971). Long-term treatment with NSAIDs in humans can lead to health issues including renal, gastric and liver damages (Bjorkman, 1998, Wallace, 1997, Wallace *et al.*, 2000).

Mammalian COX-homologues have been characterized in several fish species. Rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*), for instance, express a COX-2 homologue in their macrophages (Zou *et al.*, 1999). COX-1 and COX-2 homologues were also cloned from brook trout ovaries (*Salvelinus fontinalis*) (Roberts *et al.*, 2000). In the Japanese medaka (*Oryzias latipes*), parental exposure to 100 ng/L ibuprofen for 132 days post-hatch led to

delayed hatching in the F1 generation, even though the eggs were transferred to uncontaminated medium. Further reproductive effects in the adult fish included vitellogenin induction in male fish, fewer broods per pair and more eggs per brood (Han *et al.*, 2010). In the zebrafish (*Danio rerio*), exposure to ≥ 10 µg/L ibuprofen for 14 days led to a change in hormone levels, notably increased concentrations of 17ß-estradiol and testosterone in female fish and decreased levels of testosterone in male fish (Ji *et al.*, 2013). Exposure to ≥1 µg/L ibuprofen for 21 days led to a decrease in egg production and, as in the study by Han *et al*., 2010, parental exposure led to delayed hatching in F1, even though eggs were transferred into clean water (Ji *et al.*, 2013).

Other mechanisms of action of NSAIDs include an inhibition of the lipooxgenase pathway or interference with G-protein-mediated signal transduction (Cashman, 1996).

To represent this pharmaceutical class in our study, we selected two of the most widely used NSAIDs in North America: naproxen and ibuprofen (Khetan *et al.*, 2007).

Antibiotics

Antibiotics are used in human and veterinary care to inhibit the proliferation of bacteria in the host. They include a variety of mechanisms of action that are specifically designed to target only bacterial components and interfere with bacterial reproduction (Sanderson *et al.*, 2004). For instance, penicillin inhibits the bacterial wall synthesis, while tetracyclins impair protein manufacture by binding to bacterial ribosomes (Corcoran *et al.*, 2010).

The development and occurrence of antibiotic resistant bacteria in receiving waters of WWTP effluents is the primary concern for this pharmaceutical class (Boxall *et al.*, 2012a). Furthermore, the toxic impact of antibiotics in fish has been evaluated in a few studies. Although aimed at bacterial targets, acute toxicity of sulfamethoxazole and trimethoprim has been observed in the high mg/L range after 48 to 96 h exposure in Japanese medaka (*Oryzias latipes*) (Kim *et al.*, 2007). Tetracyclins have been shown to suppress macrophage and leukocyte responses in fish at environmental levels (0.1-50 µg/L), as well (Grondel *et al.*, 1985, Wishkovsky *et al.*, 1987).

We selected erythromycin, sulfamethoxazole and trimethoprim in our study to represent this pharmaceutical class.

Lipid Regulators

Antilipidemic drugs decrease the concentration of cholesterol and triglycerides in the blood plasma by promoting hepatic uptake and metabolism of free fatty acids (Martin *et al.*, 1997, Spencer *et al.*, 1996). Lipid regulators can be distinguished into statins and fibrates, with the latter receiving more attention in environmental research (Fent *et al.*, 2006). Fibrates activate the gene transcription of lipid regulatory proteins by binding to peroxisome proliferator-activated receptors (PPARs) (Staels *et al.*, 1998). PPAR genes are also present in fish and have been described in Atlantic salmon (Ruyter *et al.*, 1997), plaice (Leaver *et al.*, 1998) and zebrafish (Ibabe *et al.*, 2002). The activation of the PPARα pathway then leads to the generation of reactive oxygen species (ROS) and oxidative stress, particularly in the liver (Devchand *et al.*, 2004).

Clofibrate exposure induced PPARγ in salmon hepatocytes (Ruyter *et al.*, 1997). It has also been associated with oxidative stress in the liver and gill of the eastern mosquito fish (*Gambusia holbrooki*) (Nunes *et al.*, 2008). ROS formation was moreover believed to be the trigger for the histopathological alterations observed in liver, kidney and gill of rainbow trout exposed to 5-100 µg/L clofibrate (Triebskorn *et al.*, 2007). Fibrate exposure has been linked with reduced plasma testosterone levels in many fish species (Mimeault *et al.*, 2005, Prindiville *et al.*, 2011, Skolness *et al.*, 2012). Exposure to 1.5 mg/L gemfibrozil for 96 h induced a 50 % reduction of plasma testosterone in goldfish. Reproductive effects are, therefore, not surprising and have been documented for both clofibrate and gemfibrozil. Fathead minnows exposed to 0.01-1 mg/L clofibrate showed impairs spermatogenesis, reduced sperm count and lower plasma androgen concentrations (Runnalls *et al.*, 2007), while exposure to 1.5 mg/L gemfibrozil reduced fecundity (Skolness *et al.*, 2012). Furthermore, clofibrate (0.1-1 mg/L) and gemfibrozil (5 mg/L) exposure correlated with changes observed in embryogenesis in zebrafish including an increase in time to hatch and a reduced consumption of the yolk by the embryo (Raldua *et al.*, 2008).

We selected the fibrate gemfibrozil in our study to represent this pharmaceutical class.

Neuroactive compounds (antidepressants, antiepileptics, stimulants)

Antidepressants are one of the most widely dispensed prescription drugs that are primarily used to treat major depressive disorder, anxiety disorders, eating disorders or chronic pain. One of the most widely prescribed classes of antidepressants are selective serotonin

reuptake inhibitors (SSRIs), which include fluoxetine and paroxetine. SSRIs act by inhibiting the pump that directs the neurotransmitter serotonin from the synaptic cleft back into the presynapse, thus increasing intracellular serotonin levels (Fent *et al.*, 2006). Serotonin is a neurotransmitter that has hormonal and neuronal functions (Fent *et al.*, 2006). In fish, serotonin is regulating feeding and breeding behavior (Mennigen *et al.*, 2010a, Mennigen *et al.*, 2008), appetite (Mennigen *et al.*, 2010b) and establishing social hierarchies (Mennigen *et al.*, 2011, Metcalfe *et al.*, 2010).

Exposure to SSRIs like fluoxetine has been linked with altered behavior including reduced locomotor activity in zebrafish larvae (Airhart *et al.*, 2007), decreased responsiveness to stimuli in Japanese medaka (Foran *et al.*, 2004) and reduced predator avoidance in fathead minnow adults and larvae (Painter *et al.*, 2009, Weinberger *et al.*, 2014). Decreased feeding and feeding behavior in fish was also commonly observed in fluoxetine concentrations between 10-100 µg/L (Gaworecki *et al.*, 2008, Mennigen *et al.*, 2009, Mennigen *et al.*, 2010b, Stanley *et al.*, 2007, Weinberger *et al.*, 2014). Effects on reproduction included decreased aggression during mating in Bluehead wrasse (*Thalassoma bifasciatum*) (Perreault *et al.*, 2003) and increased aggression in fathead minnows (Weinberger *et al.*, 2014), reduced fecundity in zebrafish (Lister *et al.*, 2009) and decreased milt production in male goldfish (Mennigen *et al.*, 2010a). Developmental abnormalities including curvature of the spine, reduced eye size and lack of fins have been demonstrated in Japanese medaka exposed to 0.1-5 µg/L fluoxetine (Foran *et al.*, 2004). Furthermore, exposure to selected SSRIs induced a reduction of the brain Na^+/K^+-ATPase activity in synaptosomes in brook trout (Lajeunesse *et al.*, 2011). The Na^+/K^+-ATPase pump counterbalances K^+ ion loss outside the synapse that is caused by serotonin reuptake (Lajeunesse *et al.*, 2011).

Antiepileptic drugs act by decreasing the overall activity of the central nervous system. One of the mechanisms of actions of these drugs is to block voltage-dependent sodium channels of excitatory neurons (Meldrum, 1995, Meldrum, 1996). No studies exist, to our knowledge, indicating the occurrence of voltage-dependent sodium channels in fish (Fent *et al.*, 2006).

Studies on the toxic effect of antiepileptic drugs in fish are rare and mainly focused on the model compound carbamazepine. A reduction in growth rate was induced by carbamazepine concentrations above 30 mg/L in zebrafish (van den Brandhof *et al.*, 2010). Behavioral

abnormalities including reduced swimming speed and feeding rate were observed in Japanese medaka at 6.5 mg/L carbamazepine (Nassef *et al.*, 2010b). Histopathological abnormalities in gill and liver structures have been observed in common carp (*Cyprinus carpio*) exposed to 20-100 µg/L carbamazepine (Triebskorn *et al.*, 2007). And lastly, injection of 12 ng/L carbamazepine in Japanese medaka eggs resulted in delayed development and increased mortality throughout embryogenesis (Nassef *et al.*, 2010a).

Stimulants interact with the central nervous system and are used mainly as performance enhancers, since they reduce fatigue and drowsiness. Caffeine is the world's most widely consumed psychoactive stimulant. It acts by temporarily binding to and antagonizing adenosine receptors in synapses, thus preventing the neurotransmitter adenosine to activate the receptors and induce fatigue and drowsiness (Fredholm, 1985).

At 1 g/L caffeine, survival of the fish pool barb (*Puntius sophore*) and Dwarf gourami (*Colisa lalia*) has been 150 min and 120 min, respectively (Prabhuji *et al.*, 1983). *In vitro*, caffeine has been also shown to inhibit fathead minnow (*Pimephales promelas*) acetylcholinesterase (AChE), the enzyme that breaks down the neurotransmitter acetylcholine in synapses (Olson *et al.*, 1980).

We selected carbamazepine, one of the most persistent PPCPs in the environment, paroxetine and caffeine in our study to represent this pharmaceutical class.

Synthetic hormones

The synthetic estrogen 17α-ethinyl estradiol (EE_2) is used in almost all formulations of combined oral contraceptive pills and therefore one of the most commonly used medications (Aris *et al.*, 2014). Further uses in humans implicate treatment against menopausal and postmenopausal syndrome, physiological replacement therapy in deficiency states, treatment of breast cancer in postmenopausal women or prostatic cancer and osteoporosis (Kuster *et al.*, 2005, Lima *et al.*, 2011, Moriyama *et al.*, 2004, Ying *et al.*, 2002). In aquaculture, EE_2 allows to develop single-sex populations of fish to optimize the growth (Korner *et al.*, 2008, Kuster *et al.*, 2005). EE_2 acts by binding to the estrogen receptor in the organism. Compared to its natural counterpart 17ß-estradiol (E_2) it has a one to two (humans) to five (fish) times higher binding affinity and is more resistant to biodegradation (Aris *et al.*, 2014).

Exposure to EE_2 can heavily influence the endocrine system of fish, namely increase plasma vitellogenin levels in male and female fish (Andersson *et al.*, 2007), promote intersex occurrence (Lange *et al.*, 2001, Parrott *et al.*, 2005), cause complete feminization in male fish (Parrott *et al.*, 2005), decrease egg and sperm production (Nash *et al.*, 2004), reduce fertility and fecundity (Nash *et al.*, 2004, Parrott *et al.*, 2005), as well as induce behavioral changes (Partridge *et al.*, 2010).

The compound 17α-ethinyl estradiol was measured in our study to represent this pharmaceutical class.

Most pharmaceuticals are synthesized for application in mammals, especially humans. Once pharmaceuticals are unintentionally released into the environment, the homology between targets of drugs in mammals and species of lower tropic levels causes, however, unintended interactions. Previous studies have shown the bioaccumulation and toxic effects of pharmaceuticals on organisms of lower tropic levels. To date, no studies exist that evaluated the ecotoxicological effects of pharmaceuticals in aquatic mammals. With a piscivorous diet, marine mammals would be exposed to pharmaceuticals and/ or their metabolites, if the fish they consumed had previously accumulated pharmaceuticals. This project intends to start the discussion of unintended effects of pharmaceuticals in a marine mammal. We selected the harbor seal as a model organism, since it is a year-round resident of the St. Lawrence Estuary.

2.2 Biology and Ecology of Harbor Seals

2.2.1 Harbor Seal in the St. Lawrence River

Harbor seals (*Phoca vitulina concolor*) are year-round residents in the St. Lawrence estuary and occur starting from Saguenay up to the Gulf of St. Lawrence (Lebeuf *et al.*, 2003, Lesage *et al.*, 2004). Haul-out sites can be found in the majority of the estuary (Savaria *et al.*, 2008). Harbor seals were thought to be threatened in the past. The main threats the St. Lawrence population was facing are (i) mortalities and other hunting-related incidences, (ii) haul-out disturbances, and (iii) contamination. Due to the proximity of their habitat to human settlements, seals are exposed to significant amounts of anthropogenic contaminants, like municipal effluents. Further challenges include newborn isolation, mortalities and other fishing-gear-related incidents, habitat changes, environmental disasters, feeding disturbances, injuries, and mortalities caused by the collision with boats (Savaria *et al.*, 2008). The current status of the population is unknown.

Adult harbor seals can attain a length of 1.85 m (6.1 ft.) and weigh around 130 kg (290 lb.). As other marine mammals, harbor seals have a thick layer of fatty tissue (blubber) under their skin to easily maintain the body temperature at 37 ˚C while at sea. Female seals generally live longer (30-35 years) than the male counterpart (20-25 years).

2.2.2 Diet and Bioaccumulation

Harbor seals are piscivorous top predators in the St.-Lawrence Estuary and Gulf. Their diet consists of mainly three fish species: Atlantic herring (*Clupea harengus*, 67.8 %), Atlantic cod (*Gadus morhua*, 15.9 %), and capelin (*Mallotus villosus*, 15.3 %) (Hammill *et al.*, 2000). They are, however, additionally known to be opportunistic feeders and further prey might include American eel (*Anguilla rostrata*), American sand lance (*Ammodytes americanus*), Rainbow smelt (*Osmerus mordax*), Shortfin squid (*Illex illecebrosus*), Atlantic tomcod (*Microgadus tomcod*), Smooth flounder (*Pleuronectes putnami*), winter flounder (*Pseudopleuronectes americanus*), and benthos (Savaria *et al.*, 2008). The type of prey has been demonstrated to change with age (Savaria *et al.*, 2008).

Compared to other seal species in the St. Lawrence Estuary and Gulf, which are the Grey seal (*Halichoerus gryups*), the Harp seal (*Phoca groenlandica*) and the Hooded seal (*Cystophora cristata*), Harbor seals consume only minor amounts (0.2 %) of the total fish preyed upon by pinnipeds in the Estuary and Gulf.

A piscivorous diet can be disadvantageous. As top predators of the marine food web, marine mammals bioaccumulate significant amounts of persistent xenobiotics through their diet. Lipophilic xenobiotics that are resistant to metabolic breakdown easily accumulate and magnify through the food web and finally be deposited in the blubber layer (MacDonald *et al.*, 2002, Mackay *et al.*, 2000). Due to relative longevity, complex mixtures of environmental pollutants can accumulate throughout their lifetime, making them the most contaminated animals on the planet (Letcher *et al.*, 2010, Muir *et al.*, 1996, Ross, 2000). More problematic are periods of energy deficiency and nutritional stress, like migration (e.g. humpback whales) and hibernation (e.g. polar bears). Lipid stores and associated pollutants are mobilized and organs can be exposed to seasonally high concentrations of pollutants (de Swart *et al.*, 1995, Debier *et al.*, 2006). Since St. Lawrence harbor seals spend most of the their time in the Estuary and do not hibernate, energy deficiency and nutritional stress concern the species only in periods of low abundance of prey. However, eating St. Lawrence river fish causes harbor seals to bioaccumulate higher PCB and organochlorine pesticide contamination levels than, for instance, grey seals that migrate out of the Estuary and therefore have access to less contaminated fish (Bernt *et al.*, 1999).

2.2.3 Reproductive Physiology and Bioaccumulation

Harbor seals of the St. Lawrence mate usually between early June and early August (Stirling, 1983). Implantation of the embryo takes place 2 to 3 months after fertilization (diapause) (Boulva *et al.*, 1979, Fisher, 1954), and gestation then proceeds for 8 to 9 months. Pups are born between mid-May and mid-June, with a birth weight of 11 kg and gain around 0.5 kg/day (Dube *et al.*, 2003). The lactation period lasts around 34 days in this area (Dube *et al.*, 2003).

Depending on sex and reproductive maturity, contaminant levels can vary significantly in seals and other marine mammal species. In pups and juvenile males and females, contaminant levels are similar and increase with age until sexual maturity (Bernt *et al.*, 1999). While male seals continue to accumulate contaminants, female seal contamination levels off to a plateau reflecting

a transplacental and transmammary transfer from the female to her young (Addison *et al.*, 1977, Addison *et al.*, 1973, Addison *et al.*, 1974, Anas *et al.*, 1970, Martineau *et al.*, 1987, Skaare *et al.*, 1990, Westgate *et al.*, 1997). Table 5 shows an example of PCB and DDT contamination at different maturity stages and between sexes of a study done on the St. Lawrence harbor seal population.

Table 7. Mean concentrations (ng/g lipid) of the sum of polychlorinated biphenyls (∑PCBs) and the sum of dichlorodiphenyltrichloroethane (∑DDT) in blubber of harbor seals from the St. Lawrence Estuary (adapted from (Bernt *et al.*, 1999))

	Mature		**Juvenile**		**Pups**	
Sex (n)	***Male*** (5)	***Female*** (12)	***Male*** (17)	***Female*** (13)	***Male*** (8)	***Female*** (9)
Age (years)	9.4 ± 3.0	7.5 ± 1.1	2.7 ± 0.7	2.1 ± 0.5	~ 2 mo.	~ 1.7 mo.
∑PCBs	53 900 ± 22 200	14 400 ± 2 400	14 800 ± 2 200	14 800 ± 2 480	18 100 ± 9 180	8 420 ± 3 240
∑DDT	8 590 ± 2 670	3 630 ± 615	4 800 ± 750	4 810 ± 712	5 050 ± 1 860	3 070 ± 901

2.2.4 Immune system of marine mammals

The immune system is a network of sophisticated biological structures (organs, tissues, cells and proteins) communicating with each other to protect the host from potentially pathogenic agents (Pathak *et al.*, 2011). In mammals it has a two-pronged strategy: the innate and the adaptive immune system. The two parts do not operate in isolation, but are well-integrated components of a single host defense system (Pathak *et al.*, 2011). Communication between immune cells is mediated by cytokines or other soluble factors (Peakman *et al.*, 2009). Several cytokine genes have been cloned and sequenced for the bottlenose dolphin (IL-1α, IL-1ß, IL-1 receptor antagonist, IL-4, IFNγ, and TNF α) (Inoue *et al.*, 2001, Inoue *et al.*, 1999a, Inoue *et al.*, 1999b, Inoue *et al.*, 1999c, Shoji *et al.*, 2001), the orca (IL-6) (Funke *et al.*, 2003), the beluga whale (IL-2 and IL-6) and the grey seal (IL-2) (St-Laurent *et al.*, 2000, St-Laurent *et al.*, 1999).

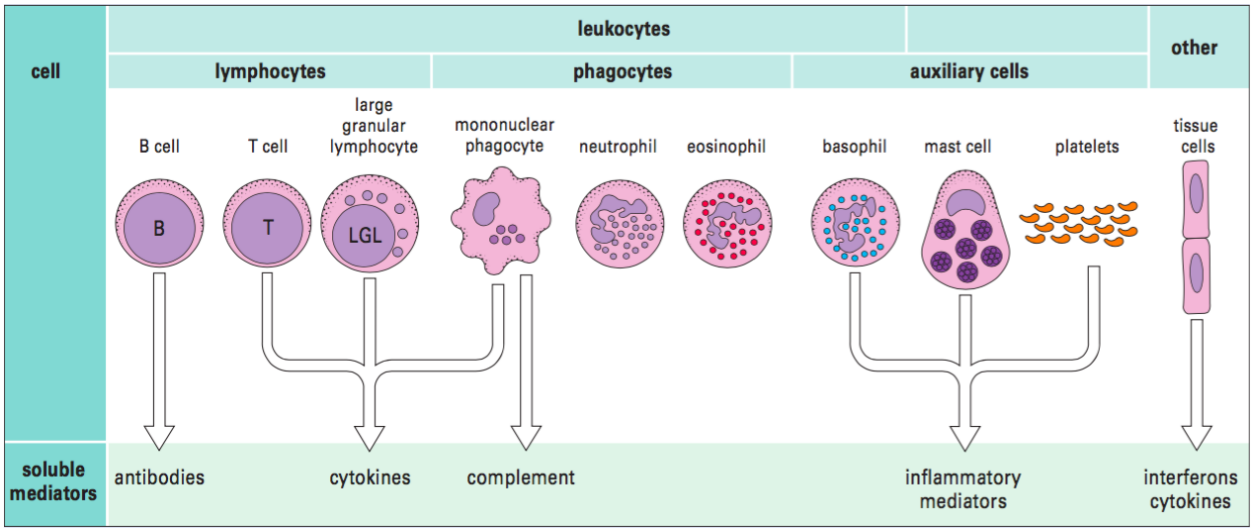


Figure 2. Overview of principal cells of the immune system and their mediators. Each cell produces and secrets only a particular set of cytokines, antibodies or inflammatory mediators. Large granular lymphocytes will be referred to as NK cells in the manuscript. Mononuclear phagocytes will be referred to as monocytes and macrophages in the manuscript (Fig. 1.1 from (Male *et al.*, 2006))

2.2.4.1 Innate Immune System

If external barriers against pathogens like skin, mucus or tears fail, organisms have internal protection to prevent infection or illness (Delves *et al.*, 2011).

The innate immunity represents the first internal line of defense and it is characterized by its presence for life, non-specificity and absence of memory (De Guise, 2004, Peakman *et al.*, 2009, van Oss, 1986). In contrast to adaptive immunity the response has always the same intensity (Male *et al.*, 2006).

Humoral Innate Immunity

The **complement system** is a group of serum proteins that support antibodies with the elimination of pathogens. Either the antibody or the pathogen can directly activate the complement protein cascade (Peakman *et al.*, 2009). The consequences of complement activation are cell lysis, production of pro-inflammatory mediators and solubilization of antigen-antibody complexes (Peakman *et al.*, 2009). Other factors include the **C-reactive protein** (CRP), which binds to bacterial cell walls and activates the complement system, **fibronectin**, which binds to both bacteria and phagocytic cells to enhance clearance, and **lysozyme**, which cleaves bacterial cell wall proteoglycans.

Cells of the innate immune system

The purpose of the cell-mediated innate immunity is to identify and eliminate pathogens either by phagocytosis and respiratory burst or by mediating the attack of larger microorganisms via attachment (Male *et al.*, 2006) . Typical cells are **phagocytic cells** (macrophages, neutrophils and eosinophils), **natural killer (NK) cells**, **dendritic cells**, **mast cells** and **basophils** (Figure 2) (Peakman *et al.*, 2009). Species specific monoclonal antibodies have been designed to identify neutrophils, eosinophils and monocytes (macrophages and dendritic cells) for marine mammals. These antibodies allow to identify the cetacean homologue surface proteins integrin ß2 (expressed by activated neutrophils and monocytes) (De Guise *et al.*, 2004) and CD11c (expressed by neutrophils and eosinophils) (Kato *et al.*, 2009).

Macrophages and neutrophils are the main phagocytic cells. Phagocytosis is a process in which foreign material is ingested. To recognize the microbe's surface, phagocytes use their pattern recognition receptors (PRRs) that then adhere with the pathogen-associated molecular patterns (PAMPs) on the microbe (Delves *et al.*, 2011). After ingestion, phagocytized particles are destroyed through a series of biochemical events named respiratory burst. Quantitative assays for the measurement of phagocytosis and respiratory burst have been developed in beluga whale (*Delphinapterus leucas*) and different seal species (De Guise *et al.*, 1995a, Frouin *et al.*, 2010b, Frouin *et al.*, 2010c, Itou *et al.*, 2001, Levin *et al.*, 2007, Noda *et al.*, 2003, Pillet *et al.*, 2000). Both phagocytosis and respiratory burst can be measured and quantified using flow cytometry. In the phagocytosis assay, leukocytes incorporate fluorescent latex beads, whereas in the respiratory burst assay, specific probes measure the formation of reactive oxygen species (ROS) (De Guise *et al.*, 1995a).

The activity of natural killer cells (NK cells) represents the first line of defense against virus infected and cancerous cells. NK induce apoptosis in nearby infected cells by releasing their granule content (House *et al.*, 2007). Unlike T cells, this cell type does not need previous sensitization (O'Shea *et al.*, 1992). NK-cell activity assays have been developed for harbor seal (de Swart *et al.*, 1994, Ross *et al.*, 1996a), grey seals (Hammond *et al.*, 2005) and beluga whales (De Guise *et al.*, 1997b). NK cell activity is measured either by flow cytometry or by ^{51}Chromium (^{51}Cr) release. For the latter, tumor cells are radiolabelled with ^{51}Cr and then incubated with peripheral blood mononuclear cells (PBMCs). Cytotoxicity is measured by

quantifying radioisotopes in the supernatant. The flow cytometric assay uses fluorescence to measure the target cell membrane integrity after NK cell incubation (De Guise *et al.*, 1997b).

2.2.4.2 Adaptive Immune System

While the innate immunity is an unspecific defense against most foreign pathogens, the adaptive immunity (also acquired immunity) is specifically tailored to individually target invading organisms that evaded the innate immune response. It is triggered and regulated by the innate immune system (Pathak *et al.*, 2011), but also directs its components e.g. complement, neutrophils, mast cells (Peakman *et al.*, 2009). Lymphocytes are the most important cell type: T cells for the cell-mediated and B cells for the humoral immunity (Pathak *et al.*, 2011).

Humoral Adaptive Immunity

B lymphocytes and especially plasma cells (differentiated B cells) generate polypeptide chains, which form antibodies (Figure 2). **Antibodies** are glycoproteins composed of two heavy and two light chains (Peakman *et al.*, 2009). They are soluble adaptor proteins with three main characteristics: they bind to a specific epitope on the invading microbe (or antigen) and then stimulate phagocytic cells or activate the complement system. Each lymphocyte and antibody is able to recognize and respond to only one specific antigen (Goldsby, 2003).

If exposed to an unknown pathogen for the first time, the system needs 7 to 10 days to reach its activity peak. The second encounter with the same antigen, then, causes a more rapid and intense response. The ability of B cells to differentiate into memory cells is the reason for this fast acting "immunological memory" (Goldsby, 2003).

Antibodies have been characterized for cetaceans as well as pinnipeds (Andresdottir *et al.*, 1987, Boyden *et al.*, 1950, Cavagnolo, 1979, Nash *et al.*, 1971, Travis *et al.*, 1972). Mono- and polyclonal antibodies have been produced to quantify antibodies in grey and harbor seals, northern elephant seal (*Mirounga angustirostris*), orcas (*Orcinus orca*), bottlenose dolphins (*Tursiops truncatus*) and sea otters (*Enhydra lutris lutris*) (Beck *et al.*, 2003, Carter *et al.*, 1990, King *et al.*, 1993, King *et al.*, 1994, King *et al.*, 1998, Taylor *et al.*, 2002).

Cells of the adaptive immune system

T lymphocytes are the cells mediating the recognition and removal of pathogens in the adaptive immune response (Figure 2). This can take place *directly* by killing the pathogens or *indirectly* by recruiting **B lymphocytes** to produce specific antibodies or activating of macrophages (Peakman *et al.*, 2009).

The direct path is mediated by the subpopulation of ***cytotoxic T lymphocytes*** (T_C, $CD8^+$T cells), which recognize their specific antigens in association with MHC I molecules (Male *et al.*, 2006). $CD8^+$T cells recognize endogenous antigens that are derived from virus infected or cancerous cells, and induce apoptosis of these cells by releasing perforin and granzymes.

The indirect pathway is conducted by the ***helper T lymphocytes*** (T_H, $CD4^+$T cells), which recognize antigens in association with MHC II molecules (Male *et al.*, 2006). $CD4^+$T cells recognize exogenous antigens that are derived from bacteria or parasites. $CD4^+$T cells can be further subdivided into T_H1 and T_H2 cells on the basis of the cytokines they produce (Male *et al.*, 2006). T_H1 cells stimulate the development of naïve T_C cells into effector and memory cells by releasing Interleukin-2 (IL-2). Furthermore, the production of the cytokine Interferon- γ (IFNγ) by T_H1 cells activates macrophages. T_H2 cells, on the other hand, produce cytokines IL-4, IL-5, IL-6 and IL-10 and stimulate B cell proliferation and antibody production (Male *et al.*, 2006).

B and T lymphocytes are morphologically similar and can be distinguished by using cross-reactive monoclonal antibodies in flow cytometry. In bottlenose dolphins, the major histocompatibility complex (MHC) class II molecules were identified on both B and T lymphocytes (Romano *et al.*, 1992). This expression pattern is similar to findings in swine (Tizard, 1992), horses (Barbis *et al.*, 1994, Crepaldi *et al.*, 1986), cats (Rideout *et al.*, 1990) and dogs (Doveren *et al.*, 1985, Doxiadis *et al.*, 1989), but differs from findings in mice in which MHC class II is only expressed by B lymphocytes (Klein, 1989), and humans in which MHC class II is expressed only by B lymphocytes and activated T cells (Abbas *et al.*, 1991, Hewitt *et al.*, 1989). Subsequently, the different subclasses of peripheral blood lymphocytes of the beluga whale (*Delphinapterus leucas*) were investigated. The cross-reactivity against beluga lymphocytes were tested for 68 bovine, ovine, murine and human antibodies of which 10 antibodies matched. Beluga whale B lymphocytes were identified by an anti-surface IgM,

since MHC class II receptors were, again, expressed by the majority of lymphocytes (De Guise *et al.*, 1997a).

Species specific mono- and polyclonal antibodies have been designed succeeding the pilot studies with cross-reactive antibodies to improve their value as diagnostic and research tools for the assessment of immune functions and health status in cetaceans. These antibodies allowed to identify the cetacean homologue surface proteins CD45R (expressed by activated T lymphocytes) (De Guise *et al.*, 1998), CD2 (expressed by T lymphocytes) (De Guise *et al.*, 2002), CD19 and CD 21 (expressed by B lymphocytes) (De Guise *et al.*, 2002) and integrin ß2 (expressed by B lymphocytes) (De Guise *et al.*, 2004).

Beyond recognizing the lymphocyte subtypes, it is also important to investigate their function. Lymphocyte proliferation is a fundamental necessity for the adaptive immune system. After stimulation with an antigen, antigen-specific lymphocytes proliferate rapidly. This reaction is essential in growing a pool of specific T cells or antibody producing B cells in response. The lymphoblastic transformation assay, also lymphocyte proliferation assay, is the most frequently utilized method to assess the immunotoxic potential of xenobiotics in cetaceans and pinnipeds (Desforges *et al.*, 2016), but has also been used to study the development of the immune system in grey seal pups (Lalancette *et al.*, 2003) and harbor seal pups (Ross *et al.*, 1994). In this *in vitro* assay, lymphocytes are stimulated with an unspecific mitogen, which stimulates the entire pool of T or B cells. Common mitogens include phytohaemagglutinin (PHA), concanavalin A (ConA), lipopolysaccharide (LPS) and pokeweed mitogen (PWM) that stimulate either T cells (PHA and ConA), B cells (LPS) or both (PWM) (de Swart *et al.*, 1993). Proliferation is then analyzed by quantifying the incorporation of labeled agents, like the radiolabeled nucleotide ^{3}H-methyl-thimidine, into the nucleus of dividing cells.

Cytokine expression of T lymphocytes after stimulation with mitogen concanavalin A (ConA) has been analyzed in the bottlenose dolphin, the beluga whale and the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*). The three species showed similar gene expression patterns with an upregulation of IL-2, IL-4, IL-13 and IFNγ mRNA, and downregulation of IL-12, IL-18 and TGFß mRNA (Sitt *et al.*, 2008).

2.2.5 Immunotoxicity in marine mammals

2.2.5.1 The immune system as a potential target for xenobiotics

Until the 1970s, toxicology studies have focused mainly on endpoints like morbidity or mortality. The first immunotoxicology review by Vos created a foundation for future research in this area (Vos, 1977). Immunotoxicity of xenobiotics can be assessed via effects in (i) histopathology of lymphoid tissues, (ii) changes in immune function and/or frequency and characteristics of immune cell populations, and (iii) changes in host resistance to infectious pathogens (Kimber *et al.*, 2002). To confirm the hypothesis that changes in certain immune parameters can lead to an overall decreased host resistance, the U.S. National Toxicology Program lead a comprehensive study on a battery of assays (Luster *et al.*, 1993, Luster *et al.*, 1992). The study confirmed a strong correlation and results corroborated the utility and sensitivity of immune endpoints as toxicity indicators.

2.2.5.2 Marine Mammal Pathologies and Epizootics

The occurrence of diseases in marine mammals residing in coastal, heavily urbanized areas raised concerns about the population's health status and fitness of the immune system of individuals. In North America, beluga whales (*Delphinapterus leucas*) displayed a variety of lesions (e.g. tumors, abscesses in the thyroid or hyperplastic nodules and serous cysts in the adrenal glands) that were linked with very high organohalogen tissue burdens (e.g. ∑PCBs: 10-500 µg/g lw) (De Guise *et al.*, 1995b, Martineau *et al.*, 1994). In the Baltic Sea in Northern Europe, pathologies like stenosis, leioma (smooth muscle tumors; frequently in the uterus), and occlusions of the uterus (closing of the uterus) were linked with high persistent organic pollutant (POPs) tissue burdens (e.g. ∑PCBs: >50-100 µg/g lw) in Baltic grey seal (*Halichoerus grypus*) and ringed seal (*Pusa hispida*) between 1970-80s. Lesions in diverse tissues, like osteoporosis and adrenal lesions (cortical hyperplasia, cortical adenomas), were also frequently found in Baltic Sea pinnipeds, later coining the term "Baltic Seal Disease Complex" (Helle, 1980, Helle *et al.*, 1976, Olsson *et al.*, 1994, Roos *et al.*, 2012). Most notably to the general public have been the reoccurring *Morbillivirus* epizootic outbreaks in 1988/89, 1990/91 and 2002 that infected and killed large proportions of the Baltic and North Sea harbor seal and Mediterranean striped dolphin (*Stenella coeruleoalba*) populations (Dietz *et al.*, 1989, Duignan *et al.*, 2014). These observations and awareness of changed health parameters in other marine organisms fueled

research to determine the reasons for deteriorated marine mammal health and the main anthropogenic stressors.

2.2.5.3 Immunotoxicity of legacy contaminants in marine mammals

The analysis of immunotoxicity in marine mammals is a relatively small field with little over 50 studies reporting effects of pollutants on parameters of the immune system (Desforges *et al.*, 2016). Pinnipeds are the group of marine mammals that has been researched the most with 29 studies compared to 18 studies on the cetacean immunotoxicity and 4 studies on polar bear immunotoxicity, likely due to the accessibility of the species. Studied xenobiotics are mainly legacy contaminants like PCBs (34 studies), other organic pollutants (e.g. organochlorines, PBDEs, PAHs, dioxin and butyltins; 24 studies), heavy metals (especially mercury; 20 studies) and perfluorinated compounds (3 studies) (Desforges *et al.*, 2016). Legacy contaminants are xenobiotics that remain in the environment after the initial source of contamination has been removed or regulated more strictly. Their chemical structure is highly resistant to metabolic breakdown, and frequently these contaminants are lipophilic (Desforges *et al.*, 2016).

Immune endpoints included immune tissue histopathology, hematology and circulating immune cell populations, lymphocyte proliferation, phagocytosis and respiratory burst, NK cell activity, antibody production and cytokine gene expression (Desforges *et al.*, 2016). Lymphocyte proliferation and phagocytosis are the most frequently analyzed endpoints (Desforges *et al.*, 2016).

Immune tissue histopathology

The tissues of the immune system can be divided into two main groups: the primary or generative immune organs and the secondary or peripheral immune organs. Primary immune organs generate naïve lymphocytes in bone marrow (B cells) and thymus (T cells), whereas secondary immune organs (lymph nodes, spleen and mucosa-associated lymphatic tissues) are the sites of the specific adaptive immune response.

Very few studies have analyzed the correlation between morphological alterations in lymphoid tissues and contaminant load in marine mammals. In Northern European harbor porpoises ∑PCB 1-26 µg/g lipid weight (lw) in blubber correlated with thymic and splenic dysfunction

(Beineke *et al.*, 2005). In Greenland polar bears ∑PCB 1-20 µg/g lw in blubber had a weak positive relationship with the amount of secondary follicles in spleen and lymph nodes (Kirkegaard *et al.*, 2005).

In vivo studies on laboratory model species have reported the following consequences for the immune system, if lymphoid tissues are affected by contamination. Significant changes in cellular and humoral immune responses have been observed, when the primary immune organs thymus or bone marrow no longer provide mature lymphocytes (House *et al.*, 2007, Silkworth *et al.*, 1985, Suh *et al.*, 2002, Thurmond *et al.*, 2000). Therefore, it has to be taken into account that the effects discussed in the subsequent categories might potentially stem from changes in lymphoid tissues.

Hematology and Circulating immune cell populations

Health of vertebrates can be assessed by determining the number and relative proportions of different leukocyte types in the peripheral blood. The baseline levels of leukocytes for free-ranging and captive marine mammals are reported in the Marine Mammal Medicine Handbook (Dierauf *et al.*, 2001).

Five studies in harbor seals found links between legacy contaminant load and alterations in circulating immune cell populations using hematology to assess immunotoxicity (de Swart *et al.*, 1994, Kakuschke *et al.*, 2011, Mos *et al.*, 2006, Reijnders, 1988, Weirup *et al.*, 2013). The circulating lymphocyte concentration was assessed in most studies, and mixed results were observed. Metal contamination (Ag, Al, Be, Cd, Ni, Pb, Ti and Zn) increased the concentration of lymphocytes in Wadden Sea harbor seals at concentrations ranging between 1-4,000 µg/L ww (Kakuschke *et al.*, 2011). PCB contaminant load increased the lymphocyte concentration in Wadden Sea seals at PCB: 7109-15,062 µg/L ww (de Swart *et al.*, 1994, Reijnders, 1988), whereas it decreased the lymphocyte concentration at lower PCB tissue levels in NE Pacific seals (PCB: 0.4-7.0 µg/L ww; (Mos *et al.*, 2006)). Further species with correlations included the Northern fur seal (*Callorhinus ursinus*, (Beckmen *et al.*, 2003)), the Stellar sea lion (*Eumetopias jubatus*, (Zenteno-Savin *et al.*, 1997)) and the bottlenose dolphin (Fair *et al.*, 2013, Schaefer *et al.*, 2011).

Next to pollutant burden factors like development, age, prior infections and stress can influence circulating leukocytes (Beckmen *et al.*, 2003, Beineke *et al.*, 2010, de Swart *et al.*, 1996, Fonfara *et al.*, 2008, Hall *et al.*, 1997, Kakuschke *et al.*, 2011, Mos *et al.*, 2006). Hematology and determining the number and relative proportions of circulation immune cell populations therefore have limitations in predicting direction and magnitude of pollutant effects on leukocytes and should be combined with further immunotoxicity tests to provide reliable results (Desforges *et al.*, 2016).

Lymphocyte proliferation

As mentioned in section *2.2.4.2 Adaptive immune system*, lymphocyte proliferation is necessary to mount a sufficient pool of antigen-specific lymphocytes after a stimulation from a pathogen.

Correlations between immune-stimulation and legacy contaminant exposure were observed for free-ranging marine mammals (Fair *et al.*, 2013, Kakuschke *et al.*, 2005, Levin *et al.*, 2005) and corroborated in *in vitro* studies (Mori *et al.*, 2008, Mori *et al.*, 2006).

Most marine mammal studies, however, reported suppressive effects on lymphocyte proliferation after contaminant exposure, notably with PCBs and mercury (Desforges *et al.*, 2016). Desforges *et al*. (2016) conducted a meta-analysis of all available published data (Figure 3 and Figure 4), revealing striking dose-response relationships of PCB and heavy metal exposure on lymphocyte proliferation despite differences in study designs, congener mixture exposures and animal life history. The half maximal effective concentration (EC_{50}) of PCBs on lymphocyte proliferation in harbor seals was $EC_{50}\ PCB = 4.67 \pm 1.76$ µg/g lw.

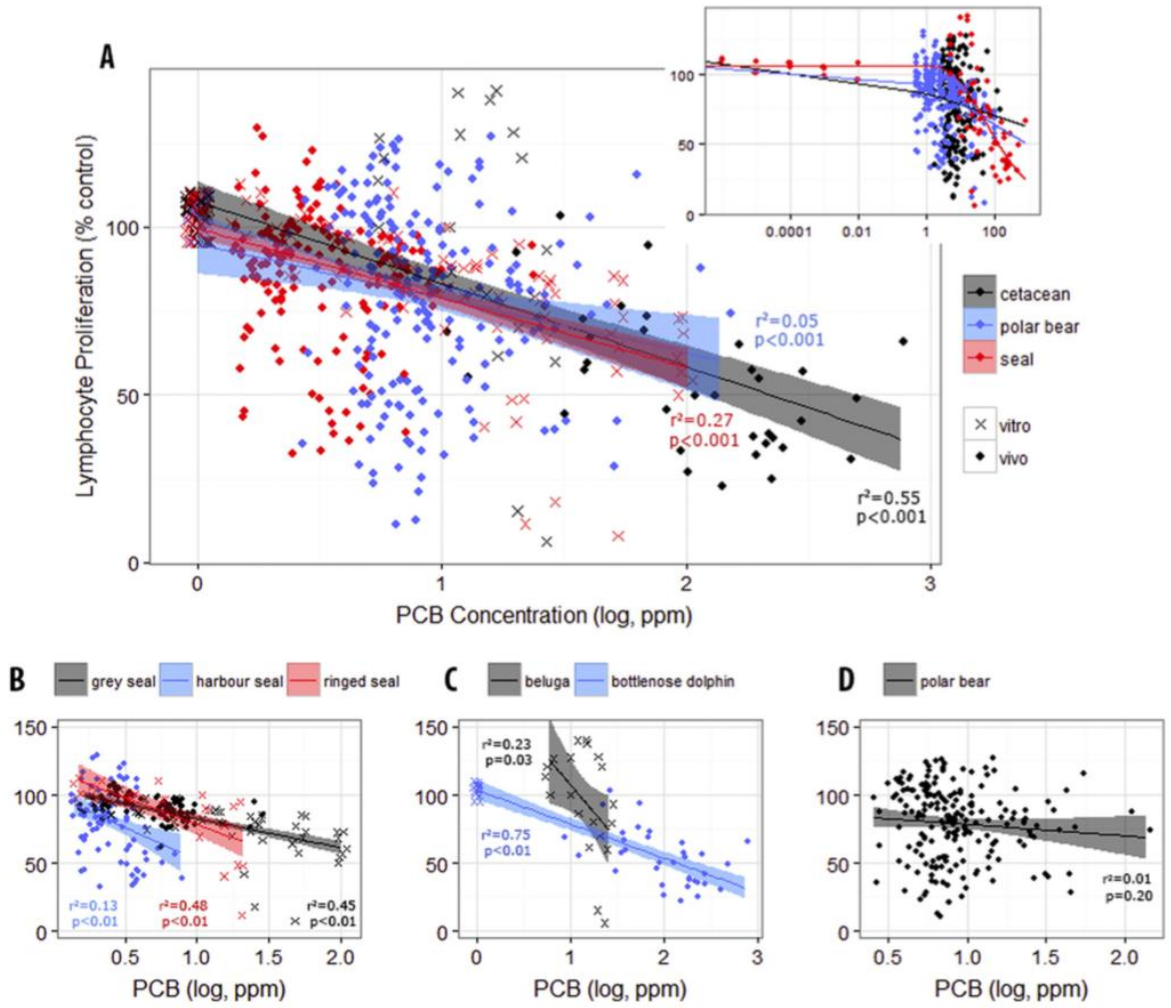


Figure 3. Reduction of lymphoproliferative response in marine mammals exposed to PCBs. The meta analysis of all present studies revealed dose-response relationships for the three clades of marine mammals (A), as well as species-specific curves for pinnipeds (B), cetaceans (C) and polar bears (D). *In vitro* (x; µg/ml) and *in vivo* (●; µg/g lw) studies have been separated. Shaded areas represent the 95% confidence intervals. (from Desforges *et al.* 2016)

Metals displayed a stronger effect towards lymphocyte proliferation with generally lower EC_{50} values than PCBs (EC_{50} *Hg* = 0.23 ± 0.31 µg/g lw, EC_{50} *MeHg* = 0.17 ± 0.12 µg/g lw and EC_{50} *Cd* = 1.83 ± 1.94 µg/g lw) (Figure 4) (Desforges *et al.*, 2016).

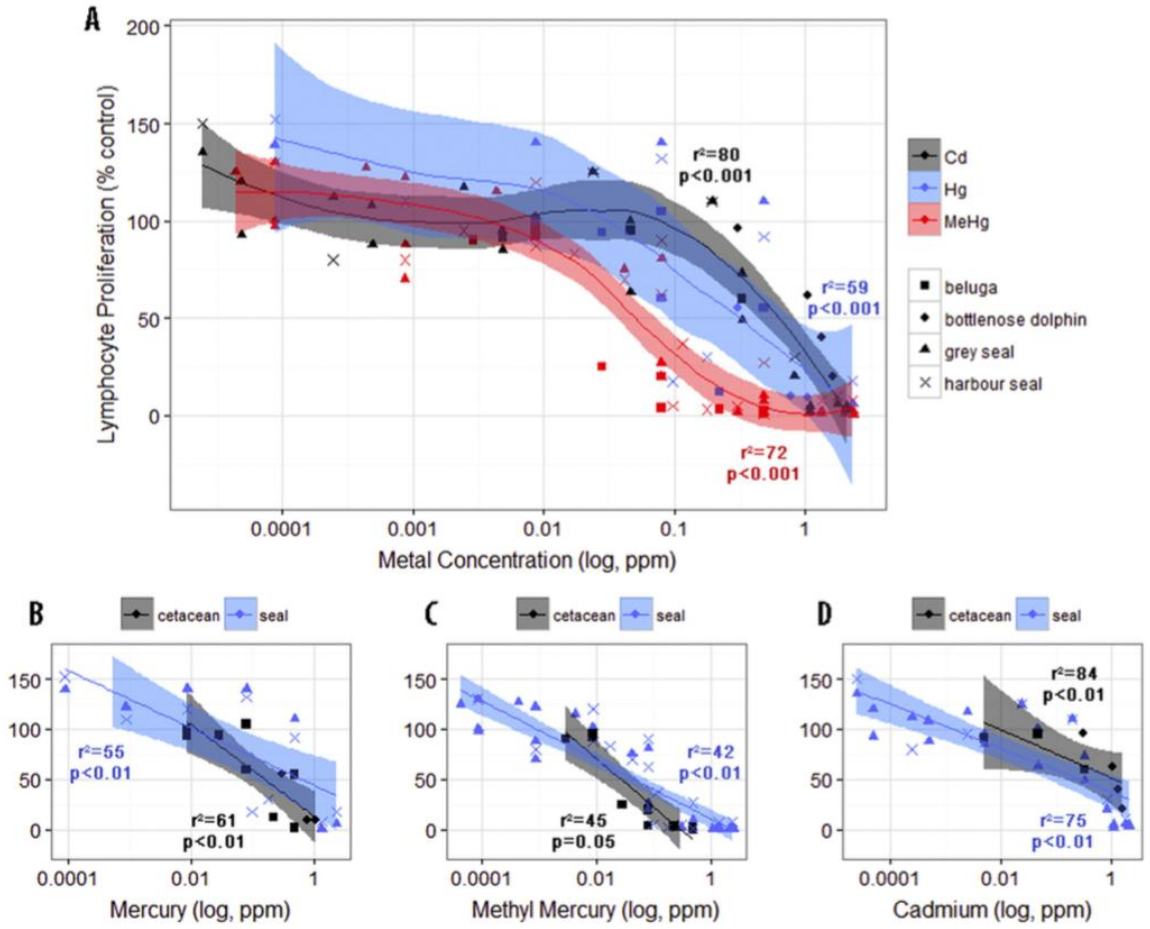


Figure 4. Reduction of lymphoproliferative response in marine mammals exposed to heavy metals. The meta analysis of all present studies revealed dose-response relationships for the mercury, methyl mercury and cadmium (A), as well as clade-specific curves for the three metals (B-D). Only *in vitro* studies were available for analysis. Shaded areas represent the 95% confidence intervals. (from Desforges *et al.* 2016)

Whereas pollutant concentration was the primary predictor of lymphocyte proliferation and was responsible for 41.4 % of the variation in case of PCBs in the meta analysis, further factors significantly influenced proliferation including pollutant type, marine mammal group and species, as well as lymphocyte subgroup (Desforges *et al.*, 2016). The model corroborated previous observations of this kind, notably the greater sensitivity of T lymphocytes compared to B lymphocytes to pollutant exposure (de Swart *et al.*, 1994, Mos *et al.*, 2006, Schwacke *et al.*, 2012, Sormo *et al.*, 2009). Whether studies were conducted *in vivo* or *in vitro* did not seem to significantly influence the result, according to the model, supporting the use of *in vitro* dose-response data in wildlife.

Phagocytosis and Respiratory burst

Phagocytosis and respiratory burst are two connected important mechanisms in the immune defense against pathogens that allow ingestion and chemical destruction of invading microorganisms (2.2.4.2). Macrophages, eosinophils and neutrophils, the most abundant leukocyte type in marine mammals, are all phagocytic cells.

The immunotoxic effects of legacy contaminants on phagocytosis were reported in 11 studies, 5 of which used harbor seal mononuclear (PBMCs) and polymorphonuclear (PMNCs) leukocytes (Desforges *et al.*, 2016). All harbor seal studies showed suppressive effects of legacy contaminants on phagocytic cells. EC_{50} values were EC_{50} *As* = 1.5 µg/mL < EC_{50} *sPCB* = 5.0 µg/mL < EC_{50} *Se* = 10.0 µg/mL < EC_{50} *Hg* = 22.0 µg/mL < EC_{50} *BDE47* = 22.6 µg/mL < EC_{50} *Zn* = 37.3 µg/mL < EC_{50} *V* = 45.4 µg/mL < EC_{50} *BaP* = 140.5 µg/mL (Desforges *et al.*, 2016, Frouin *et al.*, 2010a, Frouin *et al.*, 2010c, Hammond *et al.*, 2005, Mos *et al.*, 2006, Pillet *et al.*, 2000). Most compounds, with exception of Zn and PCB, were assessed only in one of the eleven study.

The meta analysis of Desforges *et al*. (2016) revealed well defined dose-response curves for PCBs, PBDEs, mercury and other metals (Figure 5). Seal phagocytic cells (EC_{50} = 3.7 ± 13.1 ppm) were found to be more sensitive to PCBs than cetacean phagocytic cells (EC_{50} = 8.2 ± 1.1 ppm). Furthermore, species specific differences in sensitivity were observed after exposure to 0.03 µg/mL Aroclor mixtures with harbor seals displaying significant reductions (20-30 %) in phagocytosis, while grey seal's phagocytic cells stayed unaffected (Hammond *et al.*, 2005).

The mechanisms of action responsible for the immunotoxic effects on phagocytic cells still need to be studied, but first indications (e.g. different sensitivities between species or congeners) point towards a receptor mediated pathway. Since planar PCBs did not influence phagocytic activity, AhR pathways can be excluded. So far, calcium mobilization, methallothioneins, cell membrane fluidity and permeability have been connected to the mediation of immunotoxic effects of non-planar PCBs and mercury on phagocytosis (Lalancette *et al.*, 2003, Pillet *et al.*, 2000).

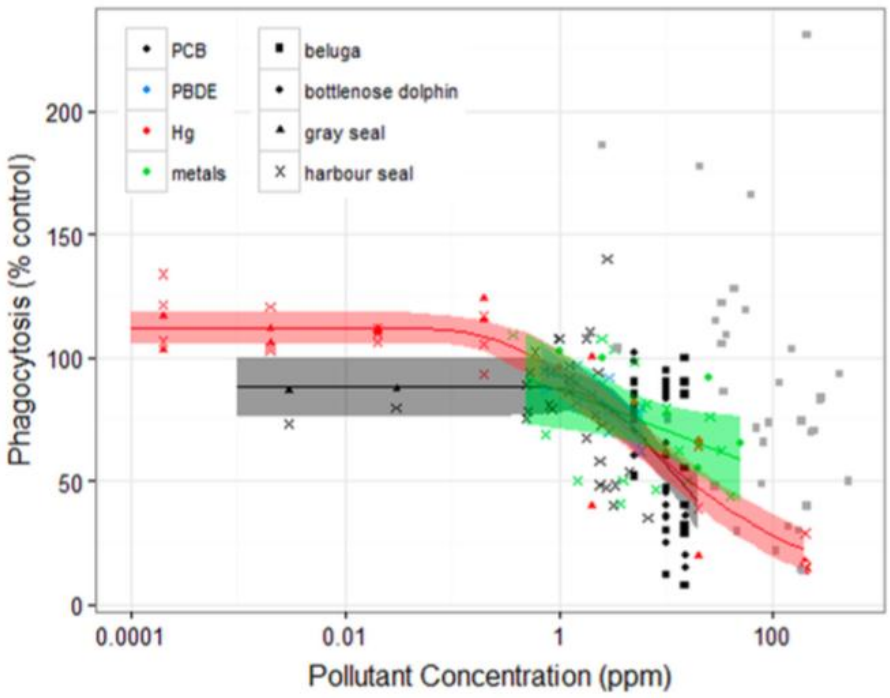


Figure 5. Reduction of phagocytosis in marine mammals exposed to PCBs. The meta analysis of all present studies revealed dose-response relationships for the major pollutant classes and species. *In vitro* (µg/ml) studies were the majority, and are not separated from the two *in vivo* studies. Shaded areas represent the 95% confidence intervals. (from Desforges *et al.* 2016)

Effects on respiratory burst were only assessed in four studies, all of which measured the reactive oxygen species (ROS) formation in harbor seals. Contaminant concentrations ranged from 0-20 µg/mL ww PCB and 0-6 µg/mL ww PBDE in harbor seal, and effects were stimulatory (Frouin *et al.*, 2010c, Mos *et al.*, 2006), suppressive (Levin *et al.*, 2004) and biphasic (i.e. hormesis) (Hammond *et al.*, 2005). Whereas a suppression of ROS formation may impair the ability of the phagocytic cell to effectively kill ingested pathogens, an excess of ROS could affect the integrity of the cell and even tissue, as well as deplete antioxidant defenses (Levin *et al.*, 2007).

The respiratory burst can be modulated through several pathways: (i) modulation of phospholipase C/ protein kinase C leading to the disruption of cellular calcium homeostasis, (ii) modulation of phospholipase A2 leading to the activation of NADPH oxidase, which stimulates ROS production, (iii) activation of tyrosine kinase activity through ROS formation, and (iv) disruption of the antioxidant defense (e.g. superoxide dismutase) (Fischer *et al.*, 1998).

NK cell activity

Although natural killer cells play an important part in the immune defense against viruses and cancer cells (2.2.4.2), only five studies have assessed the effect of contaminant load in marine mammals on NK cell activity (Fair *et al.*, 2013, Hammond *et al.*, 2005, Ross *et al.*, 1996a, Wirth *et al.*, 2014, Wirth *et al.*, 2015). The only study showing a suppression of NK cell activity was a long-term captive feeding experiment of 22 juvenile harbor seals fed herring from the relatively polluted Baltic sea. PCB contamination load ranged between 7-15 µg/mL in seals (Ross *et al.*, 1996a). Subsequent studies from other research groups failed to find changes in NK cell activity in harbor seals and other marine mammal species, potentially due to lower exposure levels, low sensitivity of NK cells to certain pollutants or differences between *in vivo* and *in vitro* studies.

Antibody production

Antibodies are essential humoral factors of the adaptive immune system, as they promote phagocytosis, NK cell activity and complement activation (2.2.4.2).

The effect of contaminant exposure on antibody production was investigated in six studies (Beckmen *et al.*, 2003, Bernhoft *et al.*, 2000, Derocher *et al.*, 2000, Fair *et al.*, 2013, Lie *et al.*, 2004), with only one focusing on harbor seals (Ross *et al.*, 1995). Most studies showed a suppression of general (total serum Ig or IgG) or specific (e.g. Ig vs. ovalbumin) antibody production with increased PCB exposure (0-15 µg/mL PCB). Mixed responses and a lack of studies limit however the possibilities to predict effects on host-resistance from antibody modulation as of today, particularly, because antibody production is a complex process being influenced by cells and organs of the immune system (Beckmen *et al.*, 2003).

Cytokine gene expression

Cytokines are proteins that enable communication of the different components of the immune system to permit a coordinated immune response (Kidd, 2003). Only six studies have assessed the effect of contamination in pinnipeds on differential cytokine expression. Cytokines analyzed in existing marine mammal studies are Interleukin (IL)-1β, IL-2, IL-4 and Transforming growth factor beta (TGFβ).

The pro-inflammatory cytokine IL-1 is strongly expressed by macrophages and has been shown to be impacted by dioxins through the Aryl hydrocarbon receptor (AhR) pathway (Monteiro *et al.*, 2008). IL-1 expression associated with PCB exposure has been analyzed in harbor and ringed seals. *In vitro*, harbor seal cells exposed to 7.2 µg/mL PCB169 or 5 µg/mL BaP showed a decrease in IL-1β expression (Neale *et al.*, 2005), whereas *in vivo*, cells of ringed seals increased expression of IL-1β in response to PCB contamination in the range of 0.1-170 µg/mL (Brown *et al.*, 2014, Routti *et al.*, 2010).

The cytokine IL-2 is crucial for leukocyte regulation, mainly T cell proliferation, NK cell activation and antibody production in B cells. The anti-inflammatory cytokine IL-4 promotes differentiation of T cells into T_h2 cells (Das *et al.*, 2008, Lehnert *et al.*, 2014, Weirup *et al.*, 2013). A study on North Sea harbor seals showed an increased expression of IL-4 and a decreased expression of IL-2 with *in vitro* exposure to 0-0.2 µg/mL MeHg (Das *et al.*, 2008). A subsequent study in North Sea harbor seals did not find effects for these cytokines with *in vitro* exposure of cells to 0-0.5 µg/mL MeHg (Kakuschke *et al.*, 2009).

Due to the lack of extensive studies on cytokine expression in immune cells of marine mammals, confounding factors influence the results to a large extend, explaining the differential results described. These include, but are not limited to, differences between *in vitro* and *in vivo* cytokine profiles and signaling, cytokine balance of individual animals that could be influenced by physiological state or infections, and lastly temporal variability of expression patterns during exposure (Das *et al.*, 2008, Fonfara *et al.*, 2008, Kakuschke *et al.*, 2009, Lehnert *et al.*, 2014, Weirup *et al.*, 2013). Nonetheless, this field should be further explored to assess the use as a tool for marine mammal immunotoxicology.

While there is growing evidence of the toxic and immunotoxic effects of legacy contaminants in marine mammals and other wildlife species over the past decades, emerging contaminants are yet to be analyzed for their immunotoxic potential in marine top predators (Daughton *et al.*, 2015). With this study we wish to initiate the research in this area.

2.2.5.4 Immunotoxicity of pharmaceuticals in organisms of lower trophic levels

The logistics of experimental design for studies in invertebrates are significantly less complex and costly than studies in free-ranging vertebrates like marine mammals. The body of literature is therefore mainly comprised of invertebrates immunotoxicity studies for emerging contaminants (Table 8).

Table 8. Immunotoxic effects observed after exposure to selected pharmaceuticals

Analyte	LOEC (mg/L)	Assay	Cell type	Species	Exposure	Reference
CAF	25-200	Proliferation	PBMCs	*H. sapiens sapiens*	66-114 h	(Horrigan *et al.*, 2006)
	7.9	Phagocytosis	Hemocytes	*E. complanata*	24 h	(Gagné *et al.*, 2006b)
CZP	14.4	Phagocytosis	Hemocytes	*E. complanata*	24 h	(Gagné *et al.*, 2006b)
	0.0001	LMS	Hemocytes	*M. galloprovincialis*	7 d	(Martin-Diaz *et al.*, 2009a)
ERY	0.00011	Phagocytosis	Hemocytes	*E. complanata*	24 h	(Gust *et al.*, 2012)
	20	Phagocytosis	Hemocytes	*M. edulis*	21 h	(Lacaze *et al.*, 2015)
EE_2	294.4	Phagocytosis	Hemocytes	*M. galloprovincialis*	0.5 h	(Canesi *et al.*, 2007b)
GEM	0.75	Phagocytosis	Hemocytes	*E. complanata*	24 h	(Gagné *et al.*, 2006b)
	2.5	Phagocytosis	Hemocytes	*M. galloprovincialis*	0.5 h	(Canesi *et al.*, 2007a)
IBU	0.5	Proliferation	Hemocytes	*R. philippinarum*	7d	(Matozzo *et al.*, 2012)
NAP	8	Phagocytosis	Hemocytes	*E. complanata*	24 h	(Gagné *et al.*, 2006b)
PAR	15	Phagocytosis	Hemocytes	*M. edulis*	21 h	(Lacaze *et al.*, 2015)
SMX	70.9	Phagocytosis	Hemocytes	*E. complanata*	24 h	(Gagné *et al.*, 2006b)
	0.00055	ROS production	Hemocytes	*E. complanata*	24 h	(Gust *et al.*, 2012)
	0.00055	COX activity	Hemocytes	*E. complanata*	24 h	(Gust *et al.*, 2012)
TMP	0.87	Phagocytosis	Hemocytes	*E. complanata*	24 h	(Gagné *et al.*, 2006b)
	<0.000022	ROS production	Hemocytes	*E. complanata*	24 h	(Gust *et al.*, 2012)
	0.00002	Phagocytosis	Hemocytes	*E. complanata*	24 h	(Gust *et al.*, 2012)
	0.005	Apoptosis and DNA damage	Hemocytes	*D. polymorpha*	1 h	(Binelli *et al.*, 2009)

Abbreviations: CAF = caffeine; COX = cyclooxygenase; CZP = carbamazepine; ERY = erythromycin; EE_2 = 17α-ethinyl estradiol: GEM = gemfibrozil; IBU = ibuprofen; LMS = Lysosomal membrane stability; LOEC = lowest observed effect concentration; NAP = naproxen; PAR = paroxetine; PBMCs = peripheral blood mononuclear cells; ROS = reactive oxygen species; SMX = sulfamethoxazole; TMP = trimethoprim.

The majority of immunotoxicity studies of our compound list was conducted on bivalves (Table 8). Phagocytosis in hemocytes was the mechanism that was tested most frequently. Since bivalves only have an innate immune system, the adaptive immune response can not be assessed in these organisms (Matozzo, 2014). Exposure time varied significantly from 0.5 h to 7 d, and therefore LOECs are varying significantly as well from 294.4 mg/L 17a-ethinyl estradiol to 0.0001 mg/L carbamazepine, respectively (Canesi *et al.*, 2007b, Martin-Diaz *et al.*, 2009a).

The pharmaceuticals selected in our study still lack a significant body of literature as this review demonstrates. With our study, we hope to diversify the information available on the immunotoxic potential of these compounds in aquatic organisms.

Section 3.

Chapter 1 – Immunotoxicity of Pharmaceuticals on a B cell lymphoma cell line

3 CHAPTER 1

IMMUNOTOXICITY OF PHARMACEUTICALS ON A B CELL LYMPHOMA CELL LINE

3.1 Introduction

In the past decades, several studies linked a decrease in immunocompetence in marine mammals to environmental contamination. Effects of legacy pollutants such as polychlorinated biphenyls (PCBs), other organic pollutants and heavy metals on the marine mammal immune system have been reviewed several times in the past decades (Beineke *et al.*, 2010, De Guise, 2004, Desforges *et al.*, 2016, Ross *et al.*, 2007, Ross *et al.*, 1996b, Ross *et al.*, 1996c). A recent comprehensive overview of over 50 marine mammal studies assessing the immunotoxic potential of legacy contaminants revealed a systemic suppression of the lymphoproliferative response and phagocytosis (Desforges *et al.*, 2016).

The decreased immunocompetence resulted in population declines and strandings (Kajiwara *et al.*, 2002, Van Loveren *et al.*, 2000), spreading of infectious diseases (Bennett *et al.*, 2001) and high prevalence in cancer in certain marine mammal species, such as beluga whales (*Delphinapterus leucas*) and California sea lions (*Zalophus californianus*) (De Guise *et al.*, 1994, Gulland *et al.*, 1996, Martineau *et al.*, 1994). Piscivorous aquatic mammals like harbor seals are particularly subject to xenobiotics because of their position in the food web, their habitat (e.g. coastal waters) where contaminant concentrations are generally higher than in the open ocean, their longevity, their adipose deposits and their specific reproductive physiology such as mother-pup transfer of contaminants (Fossi *et al.*, 2003, Ross *et al.*, 1996b). Furthermore, harbor seals are non-migratory, allowing comparisons between sites with different contamination levels (Frouin *et al.*, 2010a).

However, experimentation with free-ranging marine mammals is problematic due to the difficulty of sampling. It has been demonstrated that capture and handling methods to collect blood resulted in elevated cortisol and aldosterone concentrations in juvenile harbor seals, and were associated with altered immunological parameters (Keogh *et al.*, 2015).

I Table 1. Overview of concentrations of the selected pharmaceuticals in the environment including concentrations in fish tissue.

	Therapeutic mode of action	Lowest c [µg/L] tested	Effluent concentration (c) [ng/L]	Concentration [µg/kg] in field-caught fish
CAF	***Stimulant***	250	60-800,000 (Verlicchi *et al.*, 2010) 315-22,000 (Gagné *et al.*, 2006a) 8,638 (Wang *et al.*, 2013)	
CZP	***Anti-convulsant*** Narcosis (membrane depolarization)	250	10-2,000 (Verlicchi *et al.*, 2010) 33-137 (Gagné *et al.*, 2006a) 1,229 (Wang *et al.*, 2013) 120-1,550 (Terzic *et al.*, 2008) 23-291 (Zuccato *et al.*, 2005)	<LOD-3.1 (fillet) (Ramirez *et al.*, 2009) <LOD-8 (liver) (Ramirez *et al.*, 2009)
ERY	***Antibiotic*** Bacteriostatic	375	40-300 (Verlicchi *et al.*, 2010) 288 (Wang *et al.*, 2013) 24-420 (Terzic *et al.*, 2008) 3-50 (Zuccato *et al.*, 2005)	
EE_2	***Oral contraceptive*** Activates the estrogen receptor	125	0.6-6,000 (Verlicchi *et al.*, 2010) 0-ND (Zuccato *et al.*, 2005)	2.32 ±1.45 µg/L (plasma) (Gelsleichter, 2009b)
GEM	***Lipid regulator*** Peroxisome proliferator-activated receptor agonist	250	100-30,000 (Verlicchi *et al.*, 2010) 59-84 (Gagné *et al.*, 2006a) 2,272 (Wang *et al.*, 2013) <3-1,700 (Terzic *et al.*, 2008)	27-90 (liver) (Ramirez *et al.*, 2009)
IBU	***Anti-inflammatory agent*** COX inhibitor	250	10-300,000 (Verlicchi *et al.*, 2010) 381-1,191 (Gagné *et al.*, 2006a) 331 (Wang *et al.*, 2013) <12-11,900 (Terzic *et al.*, 2008) 13-121 (Zuccato *et al.*, 2005)	
NAP	***Nonsteroidal anti-inflammatory agent*** COX-1 and -2 inhibitor	250	10-20,000 (Verlicchi *et al.*, 2010) 217-325 (Gagné *et al.*, 2006a) 73 (Wang *et al.*, 2013) <9-1,550 (Terzic *et al.*, 2008)	
PAR	***Anti-depressant*** Selective serotonin reuptake inhibitor	250	ND (LOD=6) (Terzic *et al.*, 2008) 2-4 (Schultz *et al.*, 2010) 1-11.7 (Lahti *et al.*, 2011) 4.3-5.2 (Zhang *et al.*, 2010)	0.55 µg/L (plasma) (Gelsleichter, 2009b) 0.48-0.58 (tissue) (Chu *et al.*, 2007)
SMX	***Antibiotic*** Tetrahydrofolate synthesis inhibitor	250	3-5,000 (Verlicchi *et al.*, 2010) ND-99 (Gagné *et al.*, 2006a) 1,152 (Wang *et al.*, 2013) 19-11,600 (Terzic *et al.*, 2008) ND-130 (Zuccato *et al.*, 2005) 80-470 (Spongberg *et al.*, 2008)	
TMP	***Antibiotic*** Dihydrofolate reductase inhibitior	250	10-6,000 (Verlicchi *et al.*, 2010) 60-70 (Gagné *et al.*, 2006a) 816 (Wang *et al.*, 2013) 35-2,550 (Terzic *et al.*, 2008) 10-190 (Metcalfe *et al.*, 2003a)	

I Table 1. ***Continued*** **- Overview of concentrations of the selected pharmaceuticals in the environment including concentrations in fish tissue.**

	C in experimentally exposed fish (compartment / exposure) [tissues: ng/g; plasma: µg/L]	Acute toxicity in fish [mg/L]	LOEC inducing immune-modulation in bilvalves and vertebrates (assay / cell type / species / exposure)
CAF	17 (tissue / reclaimed water for 7 d) (Wang *et al.*, 2013)	80-120 (48 h LC50 / *P. promelas*) (Moore *et al.*, 2008)	25-200 µg/mL (lymphocyte proliferation / PBMCs / human / 66-114 h) (Horrigan *et al.*, 2006) 7.9 mg/L (phagocytosis / hemocytes / *E. complanata* / 24 h) (Gagné *et al.*, 2006b)
CZP	1.8 (tissue / reclaimed water for 7 d) (Wang *et al.*, 2013)0.3-1.0 (plasma / UTSE for 14 d) (Fick *et al.*, 2010a)	35.4 (48 h LC50 / *O. latipes*) (Kim *et al.*, 2009) 45.87 (96 h LC50 / *O. latipes*) (Kim *et al.*, 2009) 19.9 (96 h LC50 / *O. mykiss*) (Li *et al.*, 2011) 86.5 (72 h EC50 / *O. mykiss*) (van den Brandhof *et al.*, 2010)	14.4 mg/L (phagocytosis / hemocytes / *E. complanata* / 24 h) (Gagné *et al.*, 2006b) 0.1 µg/L (lysosomal membrane stability / hemocytes / *M. galloprovincialis* / 7 d) (Martin-Diaz *et al.*, 2009a)
ERY		>100 (96 h LC50 / *O. latipes*) (Kim *et al.*, 2009)	0.11 µg/L (phagocytosis / hemocytes / *E. complanata* / 24 h) (Gust *et al.*, 2012) 20 mg/L (phagocytosis / hemocytes / *M. edulis* / 21 h) (Lacaze *et al.*, 2015)
EE_2	380 (bile / 50 µg/L EE_2 for 46 h) (Larsson *et al.*, 1999)	1.6 (96 h EC50 / *O. mykiss*) (Schweinfurth *et al.*, 1996)	294.4 mg/L (phagocytosis / hemocytes / *M. galloprovincialis* / 0.5 h) (Canesi *et al.*, 2007b)
GEM	210-400 (plasma / UTSE for 16 d) (Brown *et al.*, 2007)	11.01 (96 h LC50 / *D. rerio*) (Henriques *et al.*, 2016)	0.75 mg/L (phagocytosis / hemocytes / *E. complanata* / 24 h) (Gagné *et al.*, 2006b) 2.5 mg/L (phagocytosis / hemocytes / *M. galloprovincialis* / 0.5 h) (Canesi *et al.*, 2007a)
IBU	10 (tissue / reclaimed water for 7 d) (Wang *et al.*, 2013) 5.5-102 (plasma / UTSE for 14 d) (Fick *et al.*, 2010a) 62-84 (plasma / UTSE for 16 d) (Brown *et al.*, 2007)	>300 (NEL / *C. vaiegatus*) (Knoll/BASF, 1995) 173 (96 h LC50 / *L. macrochirus*) (Knoll/BASF, 1995) >100 (96 h LC50 / *O. latipes*) (Pounds *et al.*, 2008)	500 µg/L (proliferation / hemocytes / *R. philippinarum* / 7d) (Matozzo *et al.*, 2012)
NAP	33-46 (plasma / UTSE for 14 d) (Fick *et al.*, 2010a) 9-14 (plasma / UTSE for 16 d) (Brown *et al.*, 2007)	560 (96 h LC50 / *L. macrochirus*) (Rodriguez *et al.*, 1992) 690 (96 h LC50 / *O. mykiss*) (Rodriguez *et al.*, 1992)	8 mg/L (phagocytosis / hemocytes / *E. complanata* / 24 h) (Gagné *et al.*, 2006b)
PAR	0.35 ± 0.1 (liver / primary-treated effluent for 3 months) (Zhang *et al.*, 2010) 0.19 ± 0.11 (brain) (Zhang *et al.*, 2010)	2 (LC50 / *L. macrochirus*) (FDA-CDER, 1996)	15 mg/L (phagocytosis / hemocytes / *M. edulis* / 21 h) (Lacaze *et al.*, 2015)
SMX		>750 (48 h LC50 / *O. latipes*) (Kim *et al.*, 2009) >562 (96 h LC50 / *O. latipes*) (Kim *et al.*, 2009)	70.9 mg/L (phagocytosis / hemocytes / *E. complanata* / 24 h) (Gagné *et al.*, 2006b) 0.55 µg/L (ROS production / hemocytes / *E. complanata* / 24 h) (Gust *et al.*, 2012) 0.55 µg/L (COX activity / hemocytes / *E. complanata* / 24 h) (Gust *et al.*, 2012)
TMP		>100 (48 h LC50 / *O. latipes*) (Kim *et al.*, 2009) >100 (96 h LC50 / *O. latipes*) (Kim *et al.*, 2009)	0.87 mg/L (phagocytosis / hemocytes / *E. complanata* / 24 h) (Gagné *et al.*, 2006b) <0.022 µg/L (ROS production / hemocytes / *E. complanata* / 24 h) (Gust *et al.*, 2012) 0.02 µg/L (phagocytosis / hemocytes / *E. complanata* / 24 h) (Gust *et al.*, 2012) 50 µg/L (apoptosis and DNA damage / hemocytes / *D. polimorpha* / 1 h) (Binelli *et al.*, 2009)

Abbreviations: d = days, UTSE = undiluted treated sewage effluents.

The interest of cell lines in environmental toxicology has been reviewed and positively assessed, mainly regarding cytotoxicity (Fent, 2001, Segner, 2004). Cell lines retain specific physiological characteristics and can be used in contaminant-specific mode of action testing. Previous studies demonstrated that the 11B7501 B lymphoma cell line is an excellent model to determine immunotoxicity in harbor seals (Frouin *et al.*, 2010a). In this study, we, thus, utilized this harbor seal cell line to determine possible immunotoxic effects of ten selected pharmaceuticals.

The majority of pharmaceuticals enter the aquatic environment due to domestic use (Comeau *et al.*, 2008). Many pharmaceuticals are not degraded in wastewater treatment plants (WWTPs) and will therefore be constantly discharged into the aquatic environment (Table 1) (Besse *et al.*, 2008, Gagné *et al.*, 2006a). These substances have been described as pseudo-persistent given their continuous introduction from WWTP effluent, therefore providing a continuous exposure for organisms in aquatic systems (Fent *et al.*, 2006, Ramirez *et al.*, 2009). Unlike compounds such as persistent organic pollutants (POPs) and heavy metals, accumulation of pharmaceutical compounds has not been studied in phocid tissues and in marine mammals in general. Data on uptake of pharmaceuticals through food chains is almost nonexistent (Boxall *et al.*, 2012b). Yet, several drugs have already been detected in aquatic organisms of specific interest including mussels and fish, which are generally part of the seal diet (Bringolf *et al.*, 2010, McEneff *et al.*, 2014).

The full range of ecotoxicological effects of pharmaceuticals on aquatic wildlife is largely unknown despite the fact that the characterization of such environmental effects has been identified as a priority (Boxall *et al.*, 2012b, Fent *et al.*, 2006, Kümmerer, 2009). Most studies demonstrating an impairment of different immune parameters caused by pharmaceuticals have focused on aquatic invertebrates and fish. For instance, in the freshwater mussel *Elliptio complanata* a treatment with antibiotics (erythromycin, ciprofloxacin and trimethoprim) caused an increase in phagocytosis (Gust *et al.*, 2012). Several psychotropic drugs (paroxetine, fluoxetine and venlafaxine) and antibiotics (sulfamethoxazole, trimethoprim and erythromycin) affected the immune system of a marine mussel (*Mytilus edulis*) after *in vitro* exposure (Lacaze *et al.*, 2015). Furthermore, adverse effects assessed by responses of biotransformation enzymes, neurotoxicity, immunotoxicity and endocrine disruption biomarkers in wild gudgeons (*Gobio gobio*) living downstream from pharmaceutical manufacture discharges have been reported (Sanchez *et al.*, 2011). Very few case studies have highlighted the importance of understanding

unintended effects of pharmaceuticals on aquatic or terrestrial birds and mammals, though diclofenac residues have been correlated with renal failure and the decline of three vulture species in Asia (Oaks *et al.*, 2004). To date, potential toxicological effects of pharmaceuticals on top-predators such as marine mammals remain largely unknown (Kleinert *et al.*, 2013). It is thus important to better understand effects and modes of action of these compounds on a marine mammal model, focusing on a critical endpoint: the immunocompetence.

Pharmaceuticals from various therapeutic classes were chosen based on their occurrence in WWTP effluents and fish tissues and their immunomodulatory potential in aquatic invertebrates and fish (Table 1). Compounds included antibiotics, psychotropic drugs, anti-inflammatory analgesics and 17α-ethinyl estradiol, the active compound of the contraceptive pill. Caffeine has also been selected, since it has been proposed as a chemical tracer for urban pollution (Chen *et al.*, 2002), and since it showed immunomodulatory potential in previous studies (Table 1).

We used the harbor seal 11B7501 B lymphoma cell line to screen these pharmaceuticals individually and as mixtures to give a first insight of the mode of action of these emerging compounds in a marine mammal immune model. Immune response modulation has been studied by cellular biomarkers such as cell viability, lymphoblastic transformation, cell cycle and apoptotic events.

3.2 Material and Methods

3.2.1 Culture conditions

The harbor seal 11B7501 B lymphoma cell line (Figure 1) (CRL-1940 purchased from ATCC, Manassas, VA) was maintained as previously described (Frouin *et al.*, 2010a). Briefly, cells were kept in RPMI-1640 medium with 10 % fetal bovine serum, 1 % Penicillin-Streptomycin and 25 mM HEPES (all from Sigma-Aldrich, Oakville, Canada) at 37 ˚C, 5 % CO_2 in a humid atmosphere. New 11B7501 stock was thawed for each repetition and cells were kept at concentrations ranging from 2×10^5 to 2×10^6 cells/mL.

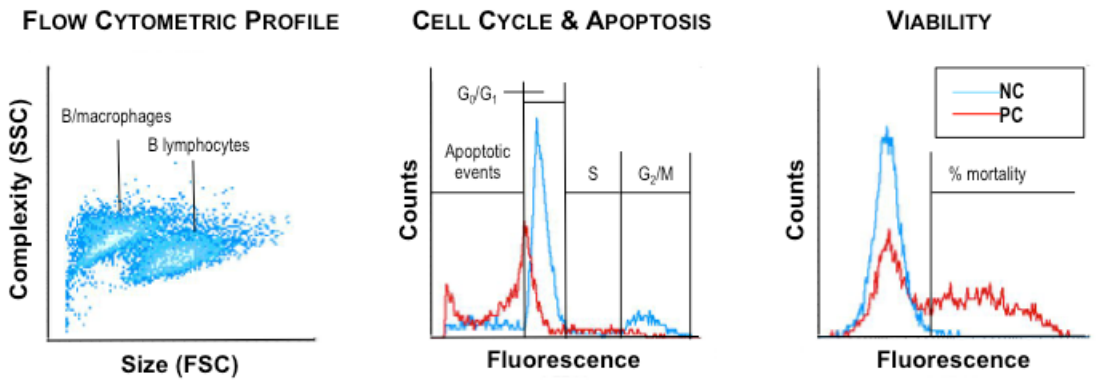
3.2.2 Preparation of xenobiotics

Compounds included antibiotics (trimethoprim (TMP), sulfamethoxazole (SMX), erythromycin (ERY)), psychotropic drugs (carbamazepine (CZP), paroxetine (PAR)), anti-inflammatory analgesics (naproxen (NAP), ibuprofen (IBU)), 17α-ethinyl estradiol (EE_2), the active compound of the contraceptive pill, blood-lipid lowering agent gemfibrozil (GEM), as well as urban tracer caffeine (CAF). Pharmaceuticals (Sigma-Aldrich, Oakville, Canada) were dissolved in DMSO, ethanol (EtOH) (both Sigma-Aldrich, Oakville, Canada) or distilled water. Final concentrations of vehicles in the samples did not exceed 0.1 %.

I Table 2. Concentration range of the individual compounds and mixtures tested. For the mixtures, two compounds were combined keeping the highest concentrations identical to the ones tested as single substances. Values are given in µg/L; concentrations that induced significant effects are in bold.

Name	Compound	Concentration range (µg/L)			
Caffeine	CAF	125	1,250	12,500	25,000
Carbamazepine	CZP	250	2,500	**25,000**	**50,000**
Erythromycin	ERY	375	3,750	37,500	75,000
Ethinyl estradiol	EE_2	125	1,250	**12,500**	**25,000**
Gemfibrozil	GEM	250	2,500	25,000	50,000
Ibuprofen	IBU	125	1,250	12,500	25,000
Naproxen	NAP	250	2,500	**25,000**	**50,000**
Paroxetine	PAR	250	2,500	25,000	50,000
Sulfamethoxazole	SMX	250	2,500	25,000	50,000
Trimethoprim	TMP	250	2,500	25,000	50,000

Name	Compound	Concentration range (µg/L)			
EE_2 + ERY	EE_2	**3,125**	**6,250**	**12,500**	**25,000**
	ERY	**9,125**	**18,750**	**37,500**	**75,000**
EE_2 + NAP	EE_2	**3,125**	**6,250**	**12,500**	**25,000**
	NAP	**6,250**	**12,500**	**25,000**	**50,000**
NAP + ERY	NAP		12,500	**25,000**	**50,000**
	ERY		18,750	**37,500**	**75,000**
NAP + CZP	NAP		**12,500**	**25,000**	**50,000**
	CZP		**12,500**	**25,000**	**50,000**



I Figure 1. Flow cytometry dot plots and fluorescent histograms of the 11B7501 cell line. Left: The flow cytometric profile shows the two distinct populations in FSC/SSC that are present in the 11B7501 cell line. Middle: Cell cycle and apoptotic events were measured as the fluorescence at 585 nm upon labeling with propidium iodide (PI). Markers were placed for the different phases of the cell cycle, and events that contained less DNA than G_0/G_1 cell were interpreted as apoptotic events. Right: Viability was assessed as the fluorescence at 679 nm upon labeling with PI. Negative control (NC, H_2O) in blue and positive control (PC, $CdCl_2$ at 50 m/L) in red.

Ten single compounds and four mixtures were tested. The full range of concentrations of single compounds and mixtures is described in Table 2.

For mixtures, the three compounds that affected at least one of the assays individually (EE_2, CZP, NAP) were selected, as well as one compound that did not affect any assay significantly (ERY).

The positive control, cadmium ($CdCl_2$), was dissolved in sterile H_2O. The concentration (50 mg/L) was chosen at a level that did not affect viability of the cell line, but influenced lymphocyte transformation and cell cycle.

3.2.3 Viability assay

Viability assays were set up in parallel to lymphocyte transformation and the cell cycle assays (3.2.4 and 3.2.5) to confirm that pharmaceutical products were not cytotoxic to the 11B7501 B lymphoma cell line.

For lymphoblastic transformation 6.25×10^5 cells were incubated with 5 µL of pharmaceutical product. For the cell cycle assay 2.5×10^5 cells were incubated with 5 µL of pharmaceutical product.

After the respective exposure time, viability of cells was evaluated by adding 0.8 µg/mL propidium iodide (PI) (Sigma-Aldrich, Oakville, Canada) to the cell suspension. A FACSCalibur (Becton Dickinson, San Jose, CA, USA) with an air-cooled argon laser providing an excitation at 488 nm was used. For each sample 5,000 events were acquired at a fluorescence emission of 620 nm (FL3). The cell population was electronically gated in a forward scatter/ side scatter (FSC/SSC) dot plot and a fluorescence frequency distribution histogram was obtained (Figure 1). The percentage of dead cells was determined as the proportion of cells with an elevated fluorescence using a marker. Data collection and analysis were performed with the CellQuest Pro software (Version 4.0.1). Results were expressed in percentage of viable cells.

3.2.4 Lymphoblastic transformation assay

For the lymphoblastic transformation assay 2.5×10^5 of cells were incubated with 2 µL of pharmaceutical product.

Change in lymphocyte proliferation after stimulation with mitogen lipopolysaccharide (LPS, from *Escherichia coli* 055:B5; Sigma, Oakville, Canada) was measured as the incorporation of methyl-^{3}H-thymidine. After cells were incubated with pharmaceuticals and 5 µg/mL LPS for 48 h, 1 micro curie (µCi) of methyl-^{3}H-thymidine (PerkinElmer, Shelton, USA) was added and cells were incubated for an additional 18 h. 96-well plates were then kept at -20 ˚C until cells were harvested onto a glass fiber filter (Tomtec Mach III Cell Harvester) and the amount of radioactive bases incorporated into the DNA was measured with a TriLux counter (Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer) and analyzed with MicroBeta Windows Workstation (Version 4.50.09, PerkinElmer, Shelton, USA) software. Raw data were expressed as counts per minute (CPM) and were then converted in percent proliferation relative to the vehicle (V, 100%).

3.2.5 Cell cycle assay

For the cell cycle assay, 2.5×10^5 cells were incubated with 5 µL of pharmaceutical product for 24 h. Medium was changed for RPMI containing no pharmaceutical agents and cells were further incubated for 72 h.

The DNA content of each cell was measured using PI. After fixation with cold 70 % EtOH, cells were washed and resuspended in a PBS solution containing PI (50 µg/mL) and RNase (100 µg/mL) (all Sigma-Aldrich, Oakville, Canada). PI also binds to double stranded regions of RNA, necessitating treatment with nucleases (Suzuki *et al.*, 1997). Therefore, fixed cells were incubated in the PI-RNase solution in the dark for 15 min at room temperature before flow cytometric analysis.

The B lymphocyte population was analyzed with a FACSCalibur (FL2, 585 nm) (Figure 1), and markers were placed for each phase of the cell cycle as well as apoptotic events (Figure 1). For each sample 5,000 events were acquired. Results were expressed in percentage of cells in different stages of the cell cycle and apoptotic events.

Approximately 20 % of events measured in the B lymphocyte gate contained a DNA mass inferior to a regular G_0/G_1 cell. These events will be referred to as “apoptotic events” and have to be distinguished from “apoptotic cells”.

3.2.6 Statistical analyses

Differences between negative controls (NC) and treated groups were evaluated by one-way ANOVA followed by Tukey’s Multiple Comparison post hoc test ($p \leq 0.05$). To further evaluate the mixtures, Pearson’s correlation analysis was used to compare the measured reduction in lymphocyte transformation and cell cycle to those values calculated from the sum of effects of mixture components. The calculations were performed using GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA).

3.3 Results

3.3.1 Lymphoblastic transformation

3.3.1.1 Single compounds

After incubation with EE_2 and NAP, proliferation of the B lymphocyte cell line decreased significantly in the two highest concentrations tested ($p < 0.0001$, $n = 12$, for both) (Figure 2A). The remaining eight single compounds did not show a negative impact on lymphoblastic transformation in the concentrations and exposure times chosen (Supplementary Figure 1).

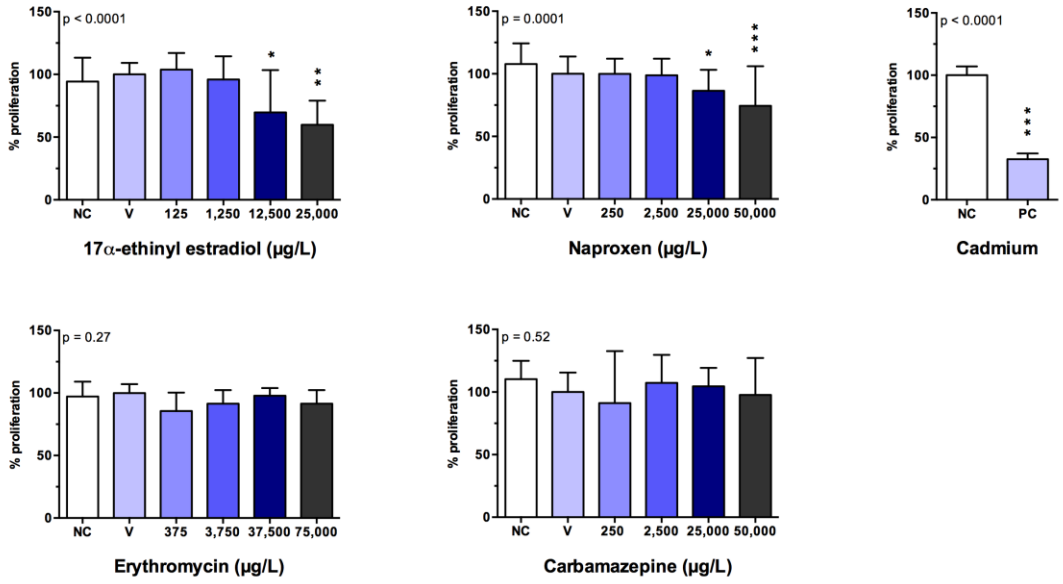
The positive control cadmium chloride (50 mg/L) showed a decrease of more than 50 % in proliferation ($p < 0.0001$, $n = 4$, Figure 2A) without affecting viability.

3.3.1.2 Mixtures

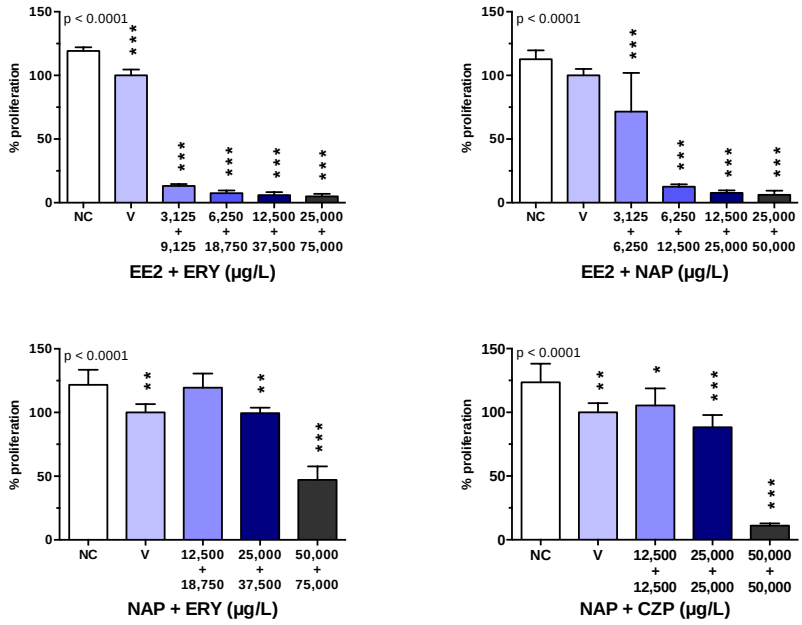
When combining two compounds, lymphoblastic transformation was inhibited to a degree greater than the simple addition of toxic effects of both chemicals individually, especially in combinations including EE_2 (Figure 2B). For the two mixtures containing EE_2, lymphoblastic transformation was inhibited significantly at all concentrations tested ($p < 0.0001$, $n = 6$, for both, Figure 2B). Proliferation levels decreased close to zero in the three highest concentrations. The remaining two mixtures also decreased lymphoblastic transformation significantly in all but one (12,500 plus 18,750 NAP+ERY) concentrations tested ($p < 0.0001$, $n = 6$, both, Figure 2B). Interestingly, the mixture that caused the most marked effect at the smallest concentration was a combination of EE_2 and ERY at 3,125 µg/L and 1,125 µg/L, respectively. ERY did not show an effect on proliferation by itself (Figure 2A), however in combination with EE_2 it turned into a potent inhibitor (Figure 2B).

No significant correlations were detected in the four mixtures (Supplementary Figure 8). These findings suggest that individual compounds in mixtures had synergistic interactions in the 11B7501 B lymphoma cell line, i.e. combined effects were greater than the sum of effects of the individual chemicals.

A – Individual compounds



B – Mixtures



I Figure 2. **Lymphoblastic transformation assay upon stimulation with the mitogen LPS following a treatment with individual pharmaceutical products (A) and mixtures (B). Results are shown as mean + SD. The vehicle control (V) represents results from samples treated with 0.1 % DMSO or EtOH, representing the vehicle concentration at the highest concentration. H_2O was used as a negative control (NC) and $CdCl_2$ was used as a positive control (PC). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.**

3.3.1.3 Viability for Lymphoblastic Transformation

Viability of B lymphocytes after 66 h incubation for the lymphoblastic transformation assay was 95.5 % ± 0.8 in the NCs. It was unaffected after incubation with any of the ten single compounds (Supplementary Figure 2).

A significant difference in viability was observed at 25,000 µg/L plus 50,000 µg/L, respectively, for the EE_2+NAP mixture (69.4 % ± 27.6) and at 25,000 µg/L plus 37,500 µg/L, respectively, for the NAP+ERY mixture (98.3% ± 1.0) ($p = 0.03$ and $p = 0.03$, respectively, $n = 4$, both, Supplementary Figure 3). The remaining mixtures did not show observable effects on viability (Supplementary Figure 3).

3.3.2 Cell cycle

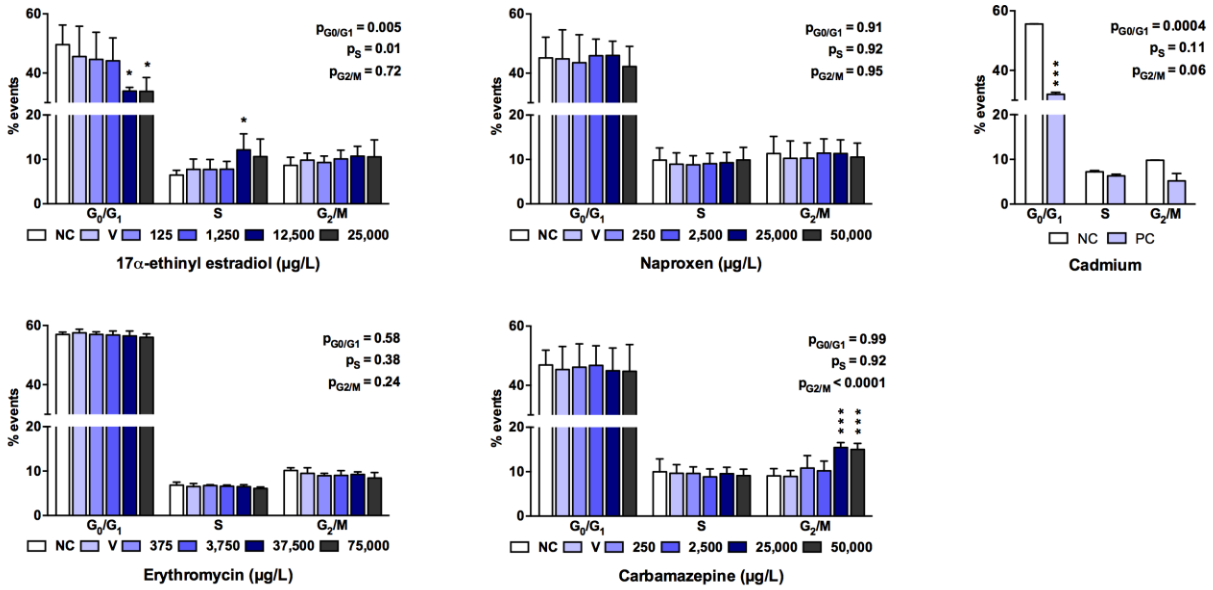
3.3.2.1 Single substances

Two out of the ten single compounds (EE_2 and CZP) showed significant changes compared to control in at least one of the phases of cell cycle (Figure 3A). In case of EE_2, the two highest concentrations (12,500 µg/L and 25,000 µg/L) showed a significant decrease in percentage of cells in the G_0/G_1 phase ($p = 0.005$, $n = 6$). Furthermore, a significant increase in percentage of cells was observed in the S phase at 12,500 µg/L ($p = 0.02$, $n = 6$), but not at 25,000 µg/L. CZP showed a significant decrease in percentage of cells in the G_2/M phase for the two highest concentrations tested ($p < 0.0001$, $n = 6$, Figure 3A).

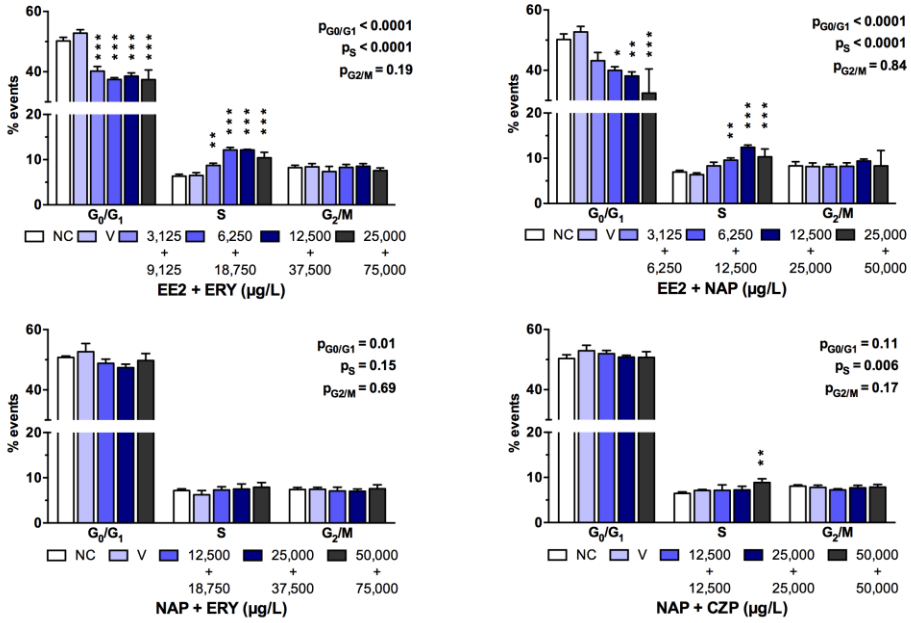
The remaining eight single compounds did not significantly affect the cell cycle in the concentrations and exposure times chosen (Figure 3A, Supplementary Figure 4).

The positive control cadmium chloride (50 mg/L) induced a significant decrease in percentage of cells in the G_0/G_1 phase ($p < 0.0001$, $n = 4$, Figure 3A), but no significant change in viability.

A – Individual compounds



B – Mixtures



I Figure 3. Cell cycle assay following a treatment with individual pharmaceutical products (A) and mixtures (B). Results are shown as the proportion of the cells in each phase of the cell cycle (mean + SD). The vehicle control (V) represents results from samples treated with 0.1 % DMSO or EtOH, representing the vehicle concentration at the highest concentration. H_2O was used as a negative control (NC) and $CdCl_2$ was used as a positive control (PC). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.3.2.2 Mixtures

When combining two compounds, the mixtures containing EE_2 show a significant decrease of cells in the G_0/G_1 phase ($p < 0.0001$, $n = 4$, for both) and a significant increase of cells in the S phase ($p < 0.0001$, $n = 4$, for both) at all concentrations tested in case of EE_2+ERY and at all but the lowest concentration tested in case of EE_2+NAP (Figure 3B). The remaining two-compound mixtures also affected the cell cycle, although to a lesser extent. In case of the NAP+CZP mixture, the percentage of cells in the S phase was increased at the highest concentration (50,000 µg/L plus 50,000 µg/L) of the mixture ($p = 0.006$, $n = 4$, Figure 3B). Combining NAP and ERY, which did not show an effect individually, resulted in a significant decrease in the proportion of cells in the G_0/G_1 phase between the vehicle control and 25,000 µg/L plus 37,500 µg/L, respectively ($p = 0.01$, $n = 4$, Figure 3B).

No significant correlations were detected in the four mixtures (Supplementary Figure 9). These findings suggest that the individual compounds in mixtures had synergistic interactions on the 11B7501 B lymphoma cell line cell cycle.

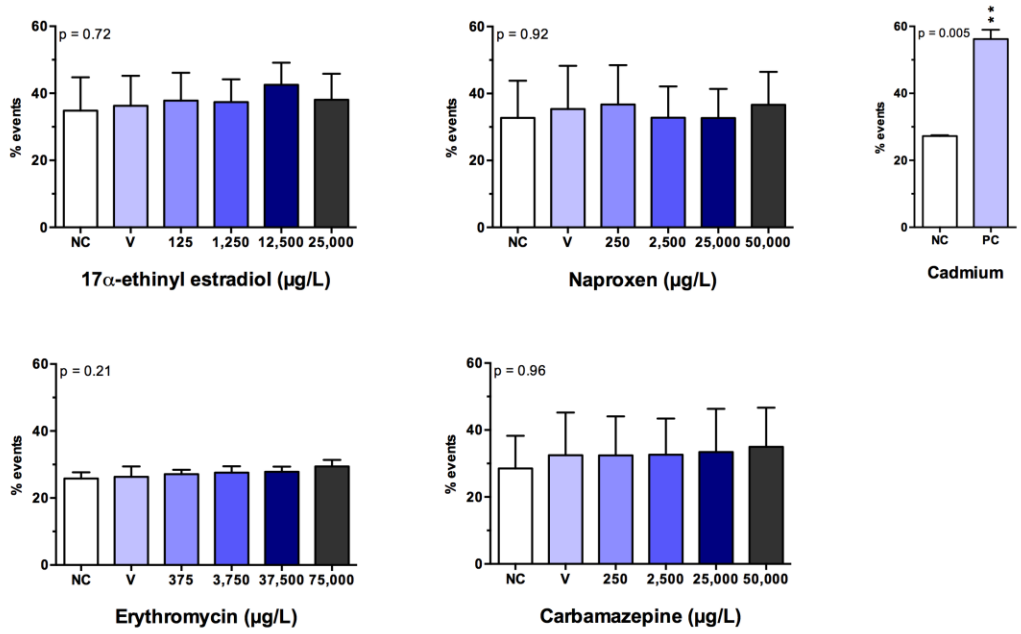
3.3.2.3 Viability for Cell Cycle

Viability of B lymphocytes after 96 h incubation for the cell cycle assay averaged at 95.0 % ± 0.6.

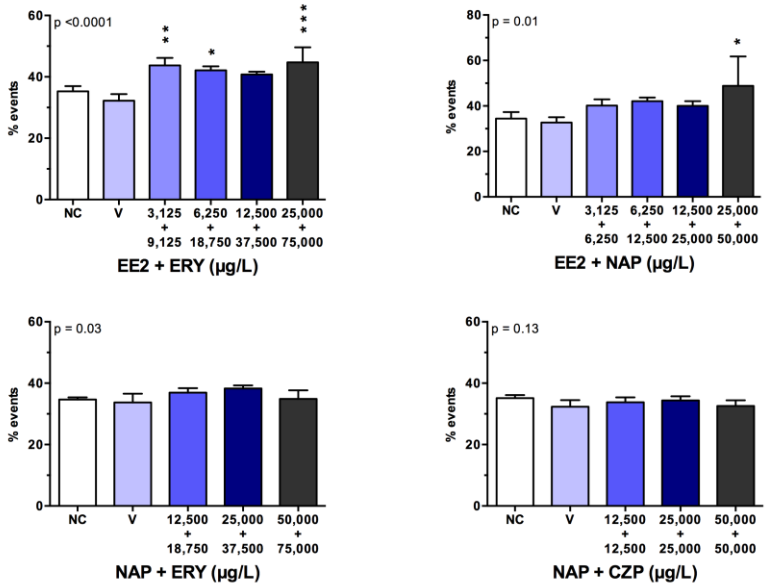
In case of EE_2, viability decreased to 88.6 % ± 9.2 in the highest concentration ($p = 0.02$, $n = 8$, Supplementary Figure 5). The remaining nine single compounds did not affect viability (Supplementary Figure 5).

The mixture EE_2+ERY decreased viability in all concentrations tested to 91.7 % ± 0.9 ($p < 0.0001$, $n = 4$, Supplementary Figure 6). The ANOVA also indicated a significant difference for the mixture EE_2+NAP ($p = 0.04$, $n = 4$, Supplementary Figure 6). The Tukey's post hoc test could not, however, identify two columns significantly differing. Viability was not affected in the remaining two mixtures (Supplementary Figure 6).

A – Individual compounds



B – Mixtures



I Figure 4. Apoptotic events measured during the cell cycle assay following a treatment with individual pharmaceutical products (A) or mixtures (B). Results are shown as the proportion of events with fluorescence below that of G_0/G_1 cells (mean + SD). The vehicle control (V) represents results from samples treated with 0.1 % DMSO or EtOH, representing the vehicle concentration at the highest concentration. H_2O was used as a negative control (NC) and $CdCl_2$ was used as a positive control (PC). * p<0.05, ** p<0.01, *** p<0.001

3.3.3 Apoptotic events

3.3.3.1 Single compounds

None of the single pharmaceutical compounds tested showed a significant change in apoptotic events (Figure 4A, Supplementary Figure 7).

The positive control cadmium chloride (50 mg/L) revealed an increase of the percentage of apoptotic events ($p = 0.005$, $n = 4$, Figure 4 A) without affecting viability.

3.3.3.2 Mixtures

In the case of the four tested binary mixtures, both mixtures containing EE_2 showed a significant increase of apoptotic events in one or more of the concentrations tested (Figure 4B). The mixture containing EE_2+ERY showed an increase in all but the 12,500 µg/L plus 37,500 µg/L concentration ($p < 0.0001$, $n = 4$), whereas the mixture containing EE_2+NAP showed a significant increase only at 25,000 µg/L plus 50,000 µg/L ($p = 0.01$, $n = 4$). The mixture of NAP+CZP did not show a significant increase of apoptotic events ($p = 0.13$, $n = 4$, Figure 4B).

No significant correlations were detected in the four mixtures (Supplementary Figure 10). These findings suggest that interactions between individual compounds in mixtures on apoptosis in the 11B7501 B lymphoma cell line were not additive.

3.4 Discussion

A recent review of marine mammal immunotoxicity research demonstrated a lack of studies assessing the immunosuppressive potential of pharmaceuticals in marine mammals. In this study we sought to assess the potential immunomodulatory or immunotoxic effects of a set of pharmaceuticals in marine mammals. To our knowledge, this is the first study that has used marine mammal immune cells to assess the immunotoxic potential of pharmaceuticals.

The most frequently utilized method to assess the immunotoxic potential of xenobiotics in marine mammals is the lymphoblastic transformation assay, also called lymphocyte proliferation assay (Desforges *et al.*, 2016). It measures the ability of lymphocytes to proliferate upon contaminant exposure. B lymphocyte proliferation is an important host defense mechanism against pathogens. After stimulation with an antigen, B lymphocytes proliferate rapidly. This

reaction is essential in growing a pool of specific antibody producing cells in response (Male *et al.*, 2006).

To complement information obtained with the lymphocyte transformation assay, we assessed the impact of the pharmaceuticals on the cell cycle. Cell cycle profiles are addressing cell behavior, the cell's capacity to respond to a stimulus and enter the process leading to cell division, or its possible engagement in the apoptosis pathway. The cell cycle consists of four distinct phases (G_1, S, G_2, M) and completes when or if cells enter a resting state (G_0). Cells increase in size (G_1), duplicate the genome (S), continue to grow (G_2) and then enter mitosis (M), separating into two daughter cells. Cells have to pass several checkpoints at G_1 ("restriction point"), G_2 ("DNA damage point") and metaphase, that monitor the cell's integrity and integrity of the DNA before and after synthesis. Cells not passing a checkpoint might be delayed in the cell cycle or might undergo apoptosis. Three types of cytotoxic effects can be observed with this assay: G_1 arrest, S slowdown with potential subsequent arrest and G_2/M arrest (Ormerod *et al.*, 1992).

Ethinyl estradiol showed significant changes in both assays without affecting viability or increasing apoptotic events. Cells seemed to be stimulated from G_0/G_1 into the S phase after exposure to EE_2. However, in the lymphoblastic transformation assay, the percentage of cells having incorporated labeled thymidine diminished. Since thymidine and other DNA bases are incorporated during the DNA synthesis phase (S phase), the lymphocyte transformation assay measures exclusively cells that have entered and successfully progressed through the S phase or beyond since addition of radiolabeled thymidine bases. An increased proportion of cells entering S phase combined with a reduced incorporation of thymidine suggest that ethinyl estradiol reduces the successful progression through the S phase and successful DNA synthesis.

Exposure to naproxen resulted in a decrease in lymphocyte transformation, while cell cycle, viability and apoptotic events remained unaffected. A potential explanation could be that exposure to NAP induced necrosis specifically in proliferating cells, which might not be detectable when assessing viability of all cells.

Exposure to carbamazepine increased the proportion of cells in the G_2/M phase, without affecting overall lymphocyte transformation, viability and the proportion of apoptotic events. This suggests that CZP possibly blocks the completion of mitosis in the cell line.

Differential exposure times and points of measurements as well as differential use of mitogen LPS across assays could also contribute to the observed discrepancies. Our results, however, elucidate the differential kinetics of the immunotoxic impact of pharmaceuticals on the 11B7501 harbor seal B lymphoma cell line. In future experiments, it would be of interest to assess kinetics of the stimulation and inhibition of lymphocyte proliferation. For this matter, the cell cycle assay should be adapted and an additional stain (bromodeoxyuridine (BrdU)) should be included in addition to PI (Ormerod *et al.*, 1992).

When comparing the lowest observed effect concentrations (LOEC) in our short-term *in vitro* study with concentrations found in plasma or tissues of fish in the environment (summarized in Table 1), we can likely exclude the possibility of acute toxic effects of individual pharmaceutical products on vertebrate immune cells *in situ*. EE_2 (LOEC = 12.500 µg/L in our study) has been found in plasma of field-caught fish at concentrations of 2.32 ± 1.45 µg/L (Gelsleichter, 2009b) and was found in bile of juvenile rainbow trout experimentally exposed to 50 µg/L EE_2 for 46 h at 380 ng/g (Larsson *et al.*, 1999). NAP (LOEC = 25.000 µg/L in our study) was found in plasma of fish experimentally exposed to undiluted sewage effluent at concentrations between 9-46 µg/L (Fick *et al.*, 2010b, Larsson *et al.*, 1999). CZP (LOEC = 25.000 µg/L in our study) can be found in concentrations between 2.3-8 µg/kg in fillet and liver of field-caught fish (Brozinski *et al.*, 2011), whereas experimentally exposed fish accumulated between 0.3-1.0 µg/L in plasma (Fick *et al.*, 2010b) and 1.6 ng/g in tissue (Bhandari *et al.*, 2011) after being exposed to undiluted sewage effluent and reclaimed water, respectively. The concentrations in plasma or tissues of fish are significantly lower than the LOEC in the harbor seal B lymphoma cell line. Biomagnification between fish and piscivorous marine mammals cannot be excluded, but the factor of accumulation still needs to be determined.

When further comparing the LOEC of individual pharmaceutical compounds in our study with half maximal effect concentrations (EC_{50}) and half maximal lethal concentrations (LC_{50}) in fish (Table 1), it is clear that at concentrations tested in the present study, mortality is a more important issue for the fish than an underlying immunotoxicity. EE_2 EC_{50} values in rainbow trout (*Oncorhynchus. mykiss*) were already observed at 1.6 mg/L after 96 h exposure (Schweinfurth *et al.*, 1996). CZP LC_{50} values ranged from 19.9 - 45.87 mg/L after 48 - 96 h of exposure in *O. mykiss* and *O. latipes* (Kim *et al.*, 2009, Li *et al.*, 2011). LC_{50} values for NAP were observed at

concentrations of 560-690 mg/L after 96 h exposure in *O. mykiss* and *Lepomis macrochirus* (Rodriguez *et al.*, 1992).

Whereas individual pharmaceutical compounds affected the cell line only in the mg/L range, the binary mixtures tested had overall more pronounced effects. Especially the mixtures containing EE_2 strongly inhibited proliferation at 66 h exposure, whereas at 24 h exposure with 72 h of latency period stimulated the cells from the G_0/G_1 phase into the S phase. This pattern of inhibition versus stimulation into the DNA synthesis phase at different exposure times was already observed to a smaller extent for EE_2 individually.

In mixtures, compounds can interact in an additive or non-additive manner (Mori *et al.*, 2006). The Pearson's correlation analysis used to assess relationships between effects of mixtures and the sum of the effects of their components indicated non-additive interactions of the pharmaceuticals in the binary mixtures, i.e. the sum of the effects of the individual chemicals was not a good predictor of the effects of a mixture. Non-additive interactions can furthermore be distinguished into synergistic and antagonistic interactions (Mori *et al.*, 2006). Since effects in mixtures seemed to increase toxicity, it can be concluded that the observed interactions were synergistic. Our findings are contrary to results obtained in invertebrates that have demonstrated that pharmaceutical mixtures can act in a non-additive but antagonistic manner on different immune parameters. Antagonistic effects were observed in a mixture of antibiotics (novobiocin, erythromycin, ciprofloxacine, oxytetracycline, sulfamethoxazole, trimethoprim) that inhibited cyclooxygenase (COX) activity in hemocytes of the bivalve *Elliptio complanata*, whereas compounds alone showed no effect (e.g. trimethoprim) or induced COX activity (ciprofloxacine and sulfamethoxazole) (Gust *et al.*, 2012). COX is involved in the production of prostaglandins, which are induced during inflammatory responses in many tissues (Gust *et al.*, 2012), as well as pathways involved in hemocyte bactericidal activity in mussels (Canesi *et al.*, 2002).

The binary mixtures assessed in our study are not as complex in compound composition as xenobiotic mixtures found in e.g. municipal effluents. However, these simple mixtures could be a first step to understanding the effects of mixtures in marine mammal immune cells before moving on to more complex and environmentally relevant mixtures in the future. Testing mixtures of compounds is more environmentally relevant than testing the toxicity or immunotoxicity of individual compounds. In the aquatic environment a multitude of

anthropogenic compounds is released by human activity including industry, agriculture and wastewater from populated areas. Wastewaters include a combination of e.g. heavy metals, organochlorines as well as pharmaceuticals, whose effects on the aquatic environment are only poorly understood (Foster *et al.*, 2014).

Lymphocytes are an essential part of the acquired immune response against pathogens and differences in proliferation after stimulation may be of concern (Mori *et al.*, 2006). In our study, lymphoblastic transformation was decreased compared to unexposed controls. A decrease in proliferation can lead to immunosuppression and increase the host's susceptibility to diseases (Imanishi *et al.*, 1980). Proliferation and lymphoblastic transformation are therefore important mechanisms to investigate in vertebrates.

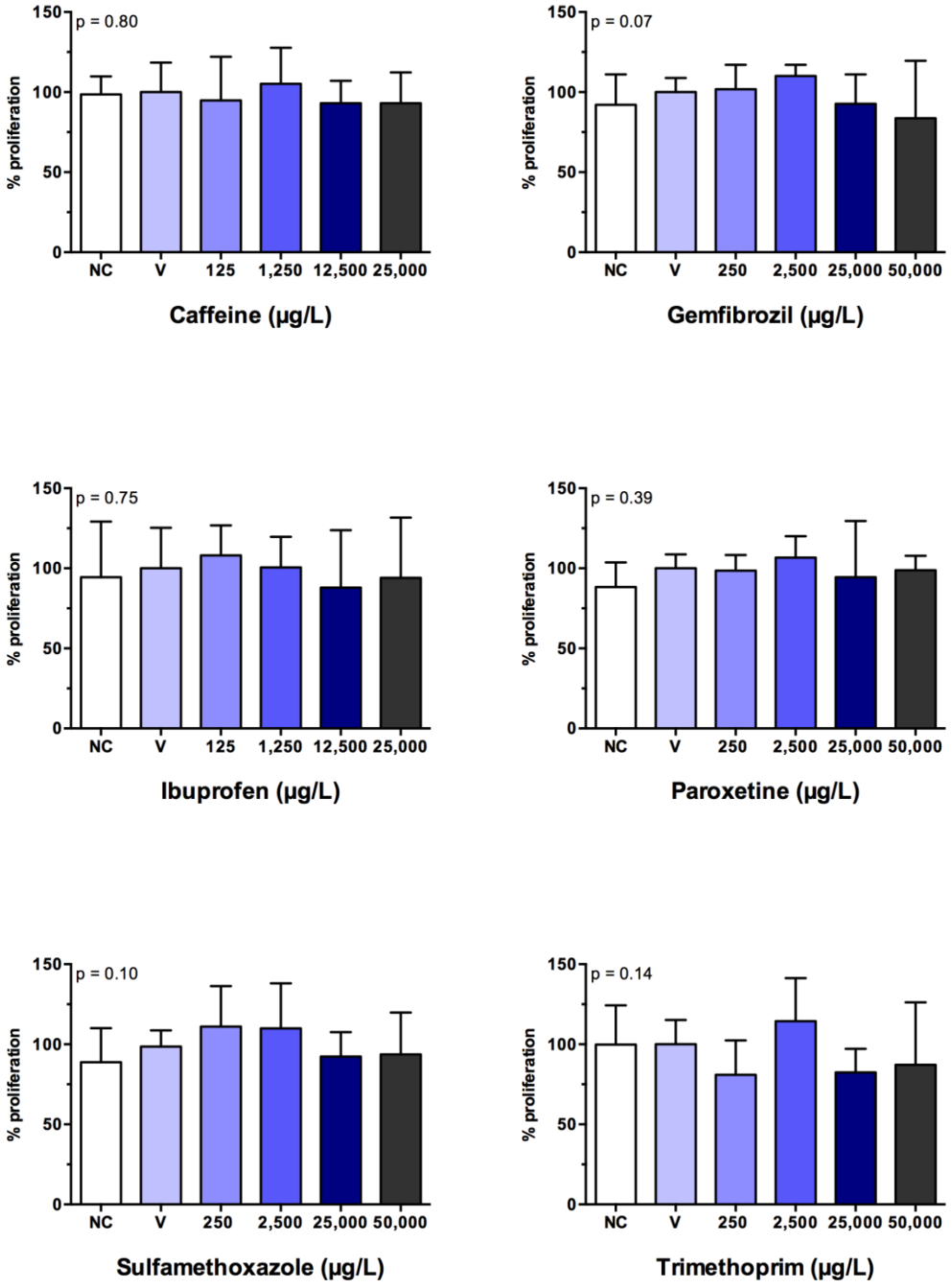
Comparison of immunotoxic effects of pharmaceuticals obtained in this study with values obtained in previous studies in bivalves and fish can be limited. The biology and ecology of bivalves and fish is very different to that of marine mammals. Their body temperature is significantly lower than that of a mammal and their feeding habits are not identical either. Mussels are filter feeders (Ismail *et al.*, 2014), whereas seals ingest most of their contaminants through their prey, which previously accumulated contaminants.

Studies on the occurrence of emerging contaminants in marine mammal tissues or plasma are sparse and very recent (Alonso *et al.*, 2015, Fair *et al.*, 2009, Gago-Ferrero *et al.*, 2013, Moon *et al.*, 2012). Synthetic musks, used as fragrances in personal care products, were the first class of compounds assessed for their bioaccumulative potential in marine mammals. Galaxolide® (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-r-2-benzopyran) was measured in blubber and liver tissue of 129 marine mammals between 1999-2006 and found at a concentration range from 2.2 to 80 ng/g lipid weight (Moon *et al.*, 2012). Triclosan, an antimicrobial additive commonly found in personal care products and toys, was found at measurable levels in plasma of 23 – 31 % of bottlenose dolphins (*Tursiops truncatus*) sampled (Fair *et al.*, 2009). Triclosan concentrations ranged of 0.025 - 0.27 ng/g wet weight. Lastly, the sunscreen agent octocrylene was detected in liver samples of the Franciscana dolphin (*Pontoporia blainvillei*) at concentrations in the range of 89 - 782 ng/g lipid weight (Gago-Ferrero *et al.*, 2013). The most polluted study site (Sao Paulo, Brazil) had a detection frequency 70 %. A subsequent study demonstrated efficient transfer of UV filters including octocrylene

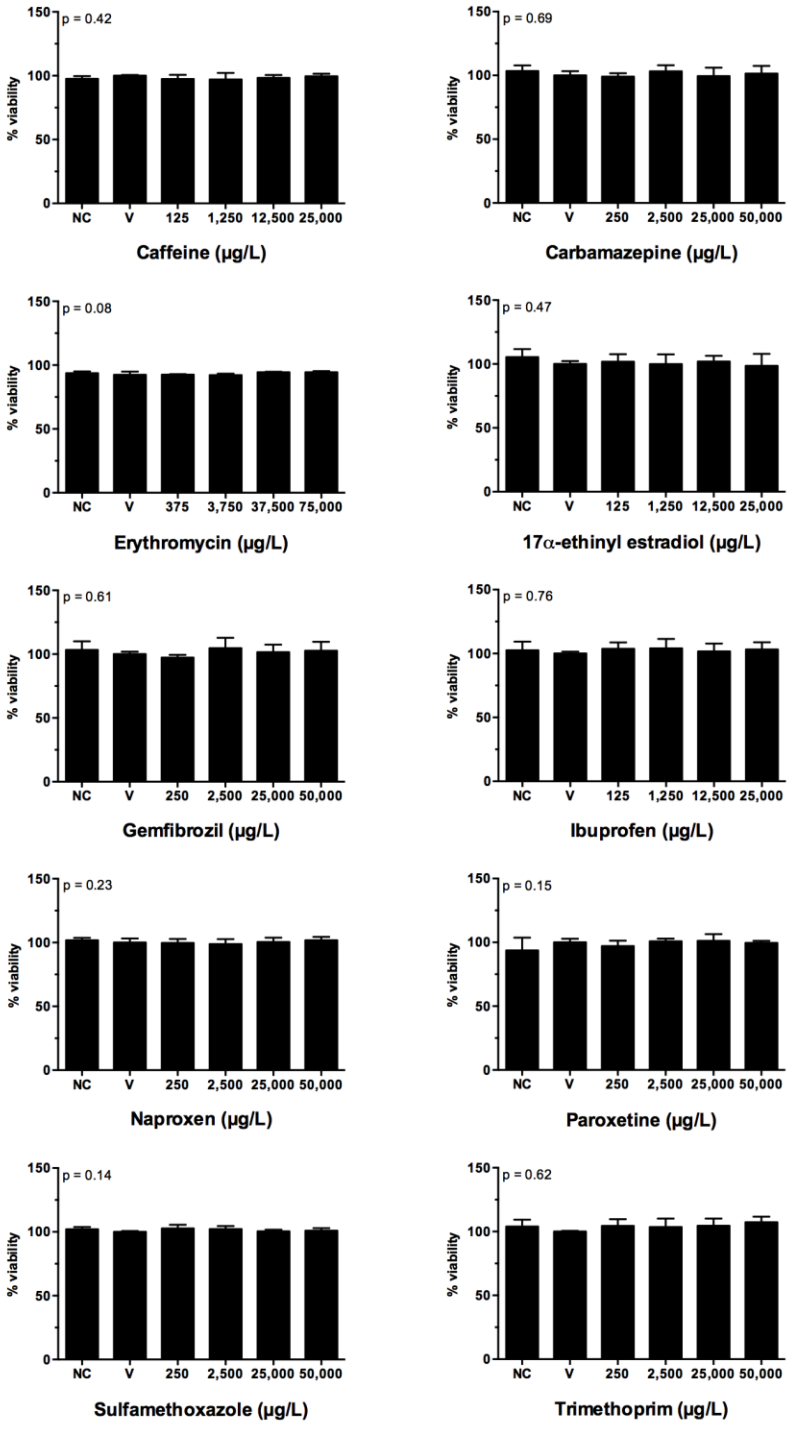
from Franciscana dolphin mothers to fetuses (F/M > 1) (Alonso *et al.*, 2015). Accumulation of pharmaceuticals has, however, not yet been assessed in marine mammals (Daughton *et al.*, 2015).

From our model and with the exposure type chosen it is difficult to extrapolate, if chronic exposure of marine mammals through their fish diet would lead to toxic or immunotoxic effects in the long term. However, the concentrations tested in this cell line were in the range of concentrations that affected fish species in previous *in vivo* exposure.

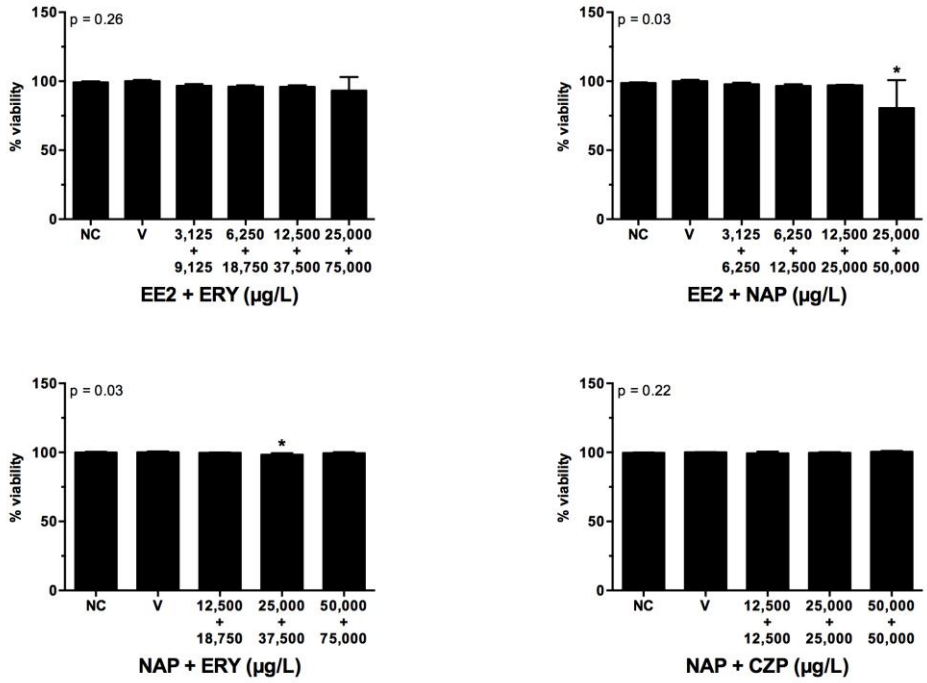
3.5 Supplementary Data



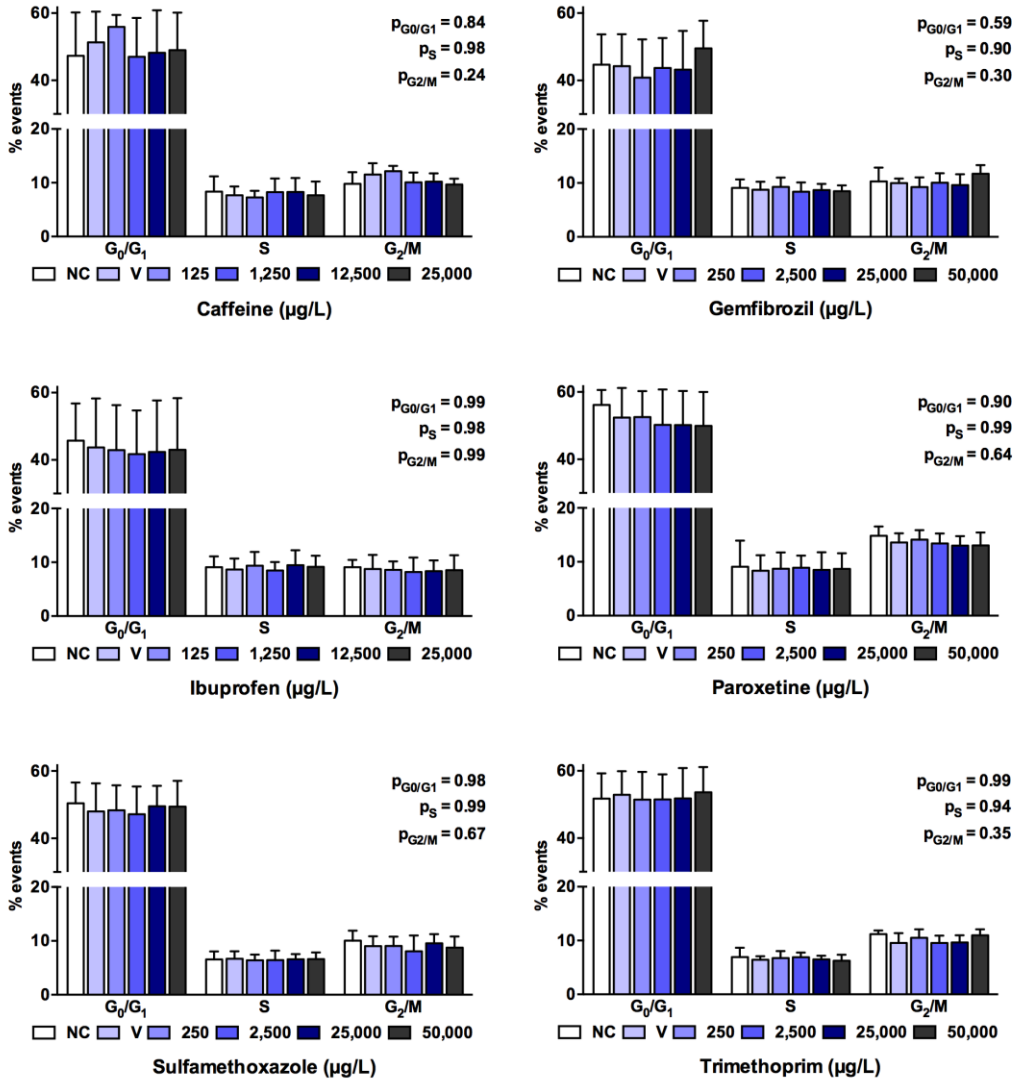
I Supplementary Figure 1. Lymphoblastic transformation assay of the six individual pharmaceutical compounds without significant effect in this study. Results are shown as mean + SD. The vehicle control (V) represents results from samples treated with 0.1 % DMSO or EtOH, representing the vehicle concentration at the maximum concentration



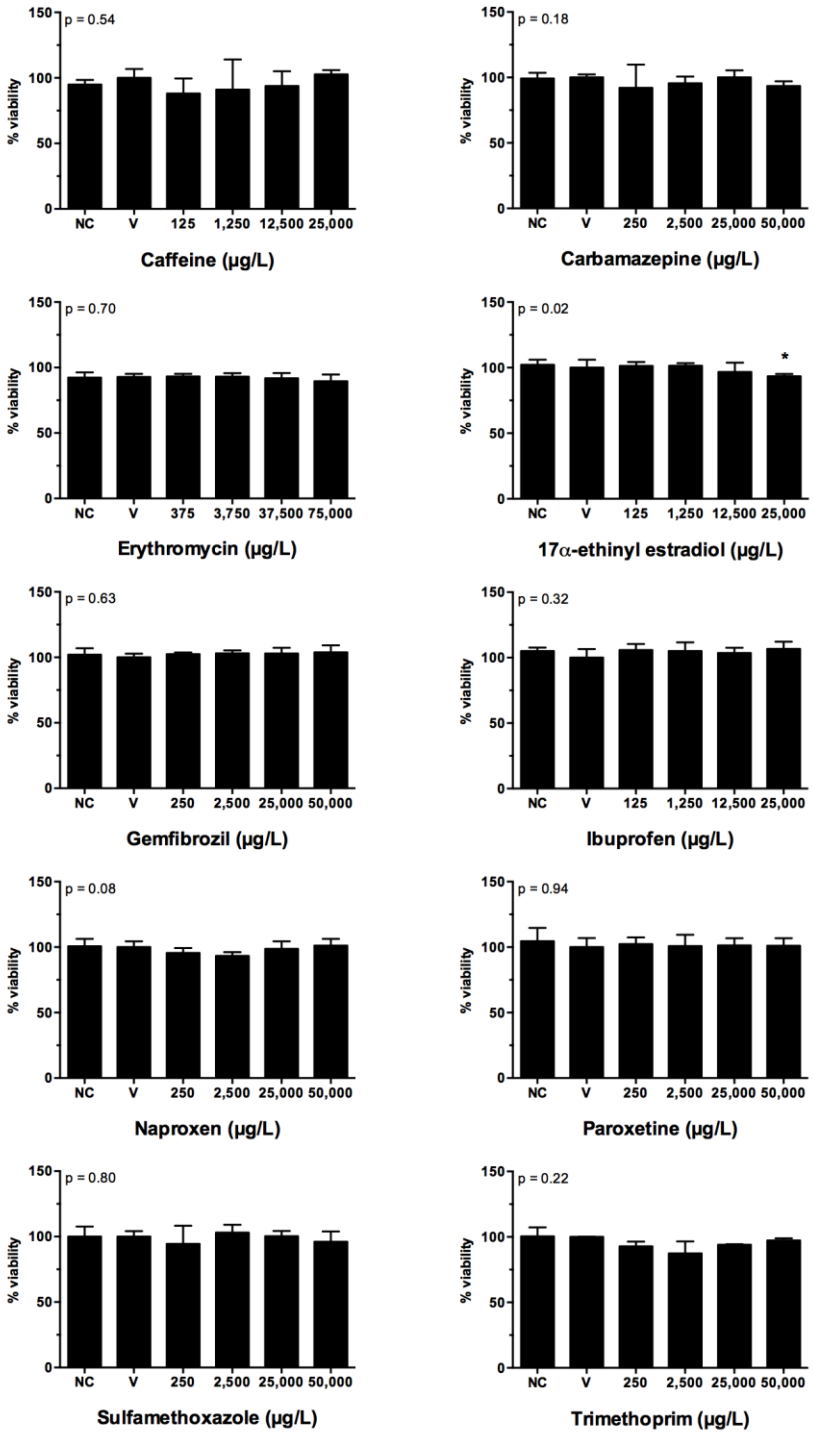
I Supplementary Figure 2. Viability assay that was set in parallel to the lymphoblastic transformation assay of the ten individual pharmaceutical compounds tested in this study. Results are shown as mean + SD. The vehicle control (V) represents results from samples treated with 0.1 % DMSO or EtOH, representing the vehicle concentration at the maximum concentration.



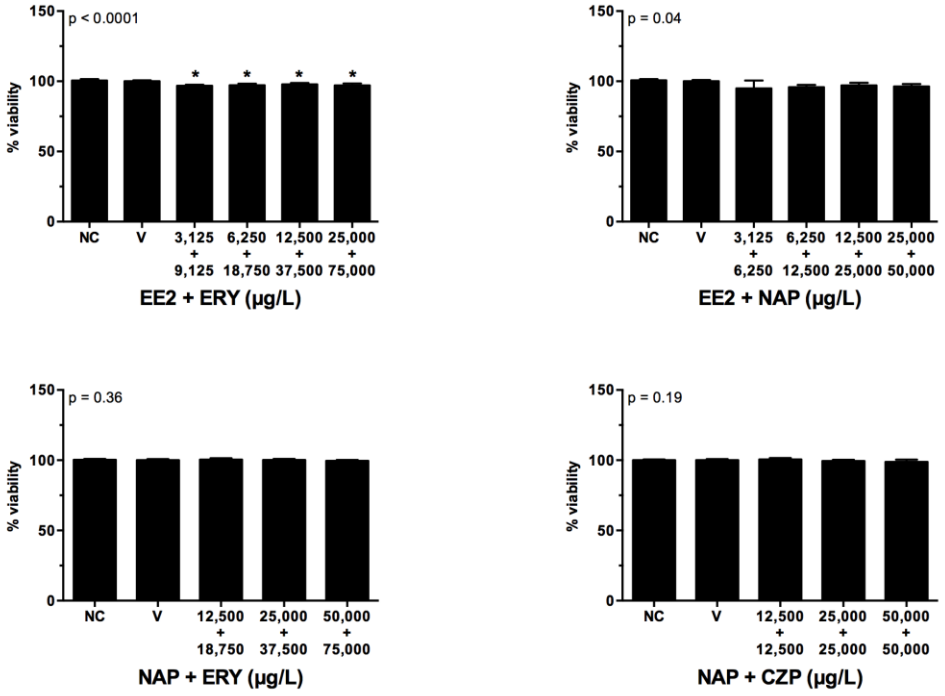
I Supplementary Figure 3. Overview of the viability assay that was set in parallel to the lymphoblastic transformation assay of the four pharmaceutical mixtures tested in this study. Results are shown as mean + SD. The vehicle control (V) represents results from samples treated with 0.1 % DMSO or EtOH, representing the vehicle concentration at the maximum concentration.



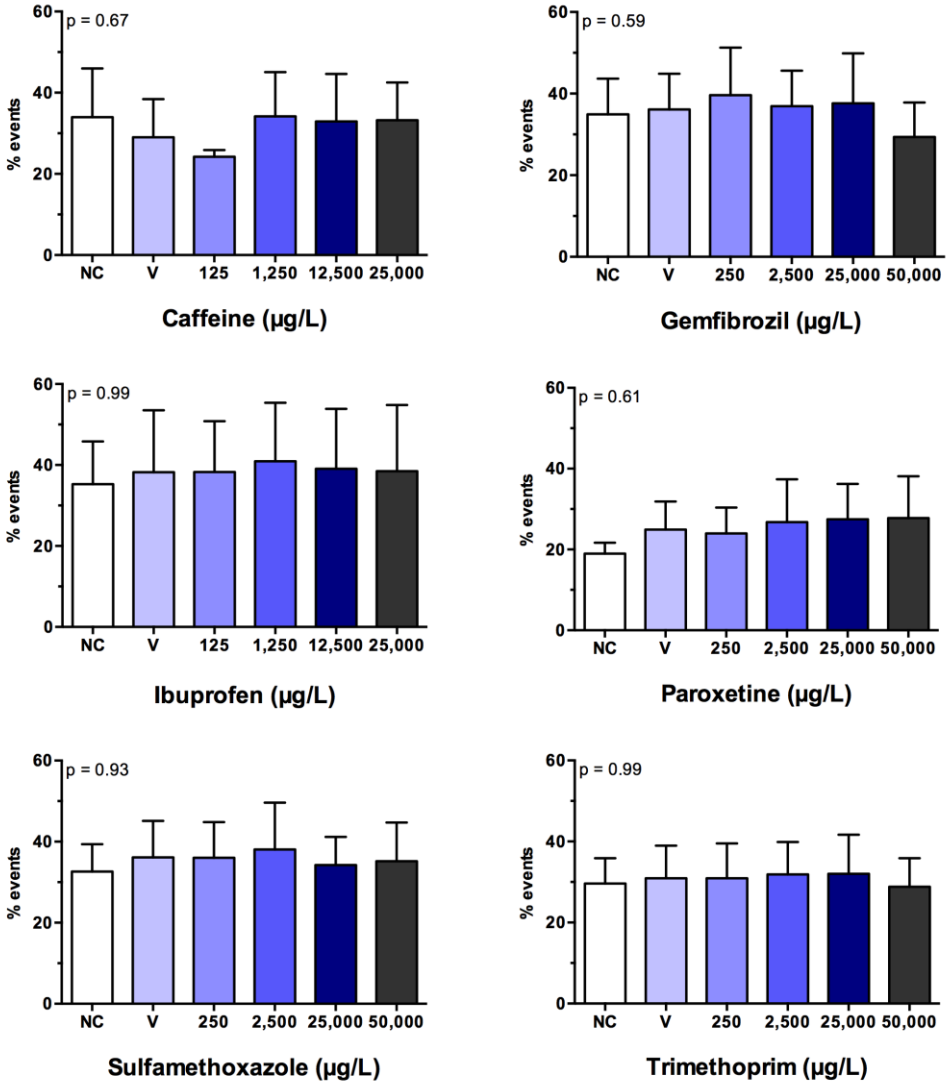
I Supplementary Figure 4. Overview of cell cycle analysis of the six individual pharmaceutical compounds without effect tested in this study. Results are shown as mean + SD. The vehicle control (V) represents results from samples treated with 0.1 % DMSO or EtOH, representing the vehicle concentration at the maximum concentration.



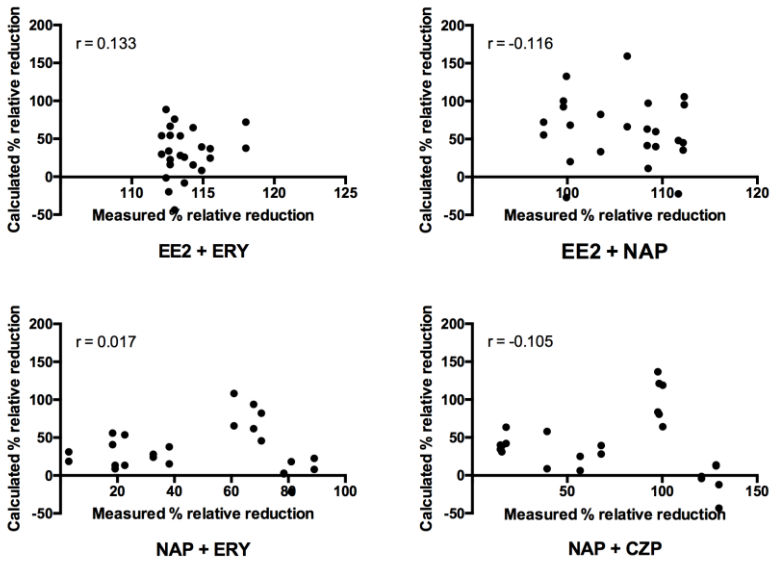
I Supplementary Figure 5. Overview of the viability assay that was set in parallel to the cell cycle assay of the ten individual pharmaceutical compounds tested in this study. Results are shown as mean + SD. The vehicle control (V) represents results from samples treated with 0.1 % DMSO or EtOH, representing the vehicle concentration at the maximum concentration.



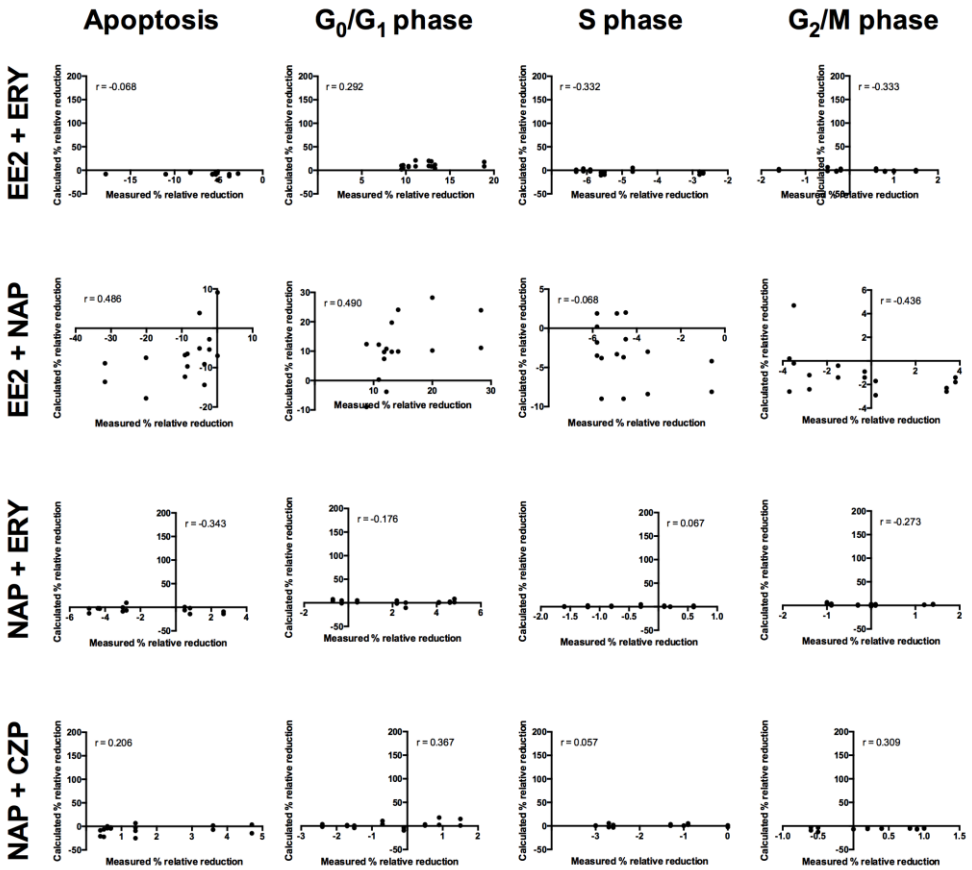
I Supplementary Figure 6. Overview of the viability assay that was set in parallel to the cell cycle assay of the four pharmaceutical mixtures tested in this study. Results are shown as mean + SD. The vehicle control (V) represents results from samples treated with 0.1 % DMSO or EtOH, representing the vehicle concentration at the maximum concentration.



I Supplementary Figure 7. Overview of the apoptotic events during the cell cycle analysis of the six individual pharmaceutical compounds without effect tested in this study. Results are shown as mean + SD. The vehicle control (V) represents results from samples treated with 0.1 % DMSO or EtOH, representing the vehicle concentration at the maximum concentration.



I Supplementary Figure 8. Pearson's correlation analysis of the four binary mixtures for the lymphoblastic transformation assay.



I Supplementary Figure 9. Pearson's correlation analysis of the four binary mixtures for the cell cycle and apoptosis assay.

Section 4.

Chapter 2 – Immunotoxicity of Pharmaceuticals on T Lymphocytes

4 CHAPTER 2

IMMUNOTOXICITY OF PHARMACEUTICALS ON T LYMPHOCYTES

4.1 Introduction

The understanding of the impacts of anthropogenic pollution on the immune system of marine mammals has gathered increased attention, due to several epizootic *Morbillivirus* outbreaks in seals and dolphins (1988/1989, 1990/1991 and 2002) (Aguilar *et al.*, 1994, Härkönen *et al.*, 2006) and increased reports of infectious diseases and cancer in marine mammals that were correlated with xenobiotic exposure (Bennett *et al.*, 2001, De Guise *et al.*, 1994, Gulland *et al.*, 1996, Martineau *et al.*, 1994). Xenobiotics have been shown to impact the immune system (Vos, 1977), which can ultimately lead to decreased host resistance (Luster *et al.*, 1993, Luster *et al.*, 1992). Past studies have evaluated the impact of xenobiotics on lymphoid tissue histology, circulating immune cells and hematology, lymphocyte proliferation, phagocytosis, NK cell activity, antibody production and cytokines in pinnipeds and cetaceans (Desforges *et al.*, 2016).

In Eastern Canada, heavy metals (Wagemann *et al.*, 1990) and organochlorines (Bernt *et al.*, 1999, Hobbs *et al.*, 2002) have been found in the aquatic environment, and accumulated in several marine mammal species. Male harbor seals from the St. Lawrence Estuary bioaccumulated 53.9 mg/kg lipid PCBs and 7.06 mg/kg lipid DDT in blubber, whereas the blubber of female harbor seals contained less PCBs (14.4 mg/kg lipid) and DDT (3.48 mg/kg lipid) (Bernt *et al.*, 1999). After the ban of PCBs in the early 1970s, a temporal study from the Canadian Arctic demonstrated a 40 % decline in PCB concentrations in Holman Island ringed seals (*Phoca hispida*) between 1972 and 1981, but no significant decline between 1981 and 1991 (Addison *et al.*, 1998). Marine mammal immunotoxicological studies have focused on these legacy contaminants because of their problematic nature and their ability to accumulate. The majority of studies investigated the mitogen-induced proliferative responses of lymphocytes. A meta analysis of all present studies demonstrated a clear dose-response relationship for the

effects of heavy metals and PCBs on the proliferative responses of lymphocytes for cetaceans and pinnipeds (Desforges *et al.*, 2016).

Next to legacy pollutants, emerging contaminants like pharmaceuticals are also present. In the St. Lawrence River, municipal effluents represent a major point source for these contaminant classes (Gagné *et al.*, 2006a). Uptake and accumulation of pharmaceutical compounds has been demonstrated in fish. Ethinyl estradiol (EE_2), the active compound of the contraceptive pill, was found at levels of up to 0.38 µg/g in bile of juvenile rainbow trout (*Oncorhynchus mykiss*) experimentally exposed to 50 µg/L EE_2 for 46 h (Larsson *et al.*, 1999). And naproxen (NAP), a commonly used analgesic and anti-inflammatory agent, was found in the range of 9-14 µg/L in plasma of rainbow trout exposed to undiluted treated sewage effluents (Brown *et al.*, 2007, Fick *et al.*, 2010a).

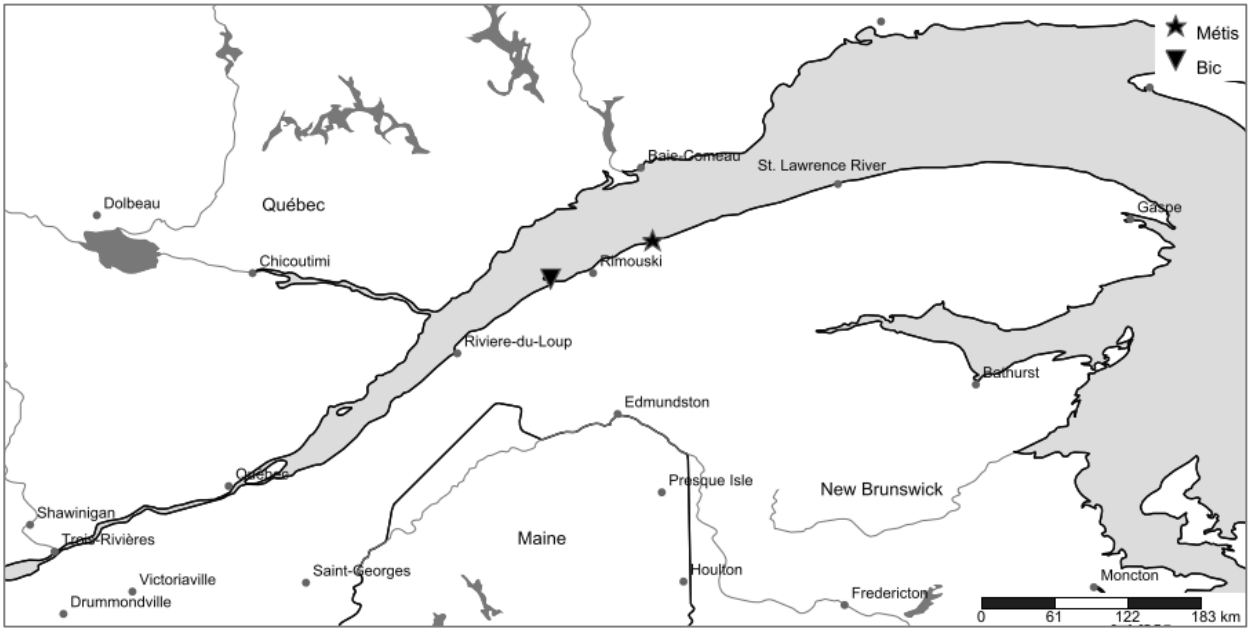
To date, there is a lack of research on the occurrence, accumulation and immunotoxic potential of pharmaceutical products in marine mammals (Daughton *et al.*, 2015). However, since fish and invertebrates accumulate certain pharmaceuticals, we hypothesize that marine mammals residing in coastal areas are exposed to these compounds through their diet. The immunotoxic potential of pharmaceuticals compounds observed in lower trophic levels, e.g. a decrease in phagocytosis in hemocytes of the Mediterranean mussel (*Mytilus galloprovincialis*) and the Eastern Elliptio (*Elliptio complanata*) induced by EE_2 and NAP exposure, respectively (Canesi *et al.*, 2007b, Gagné *et al.*, 2006b), led us to investigate immunotoxicity of pharmaceuticals in a marine top predator.

A previous study tested 10 individual pharmaceuticals and four binary mixtures in a harbor seal 11B7501 B lymphoma cell line *in vitro* using proliferation, cell cycle and viability assays (Kleinert et al., 2016a, unpublished). Proliferation and cell cycle were impacted by three of the ten individual compounds and all four binary mixtures. In the present study, we further investigated the immunotoxic potential of these pharmaceuticals on mitogen-induced proliferation in harbor seal pup peripheral blood mononuclear cells (PBMCs). The harbor seal pup is relatively immunocompetent compared to carnivorous terrestrial offspring (Ross *et al.*, 1996c) making extrapolation to adult seals easier. The aim of the present study is to establish dose-response curves to quantify the immunotoxicity of pharmaceuticals and compare the results to those using a cell line.

4.2 Material and Methods

4.2.1 Blood Sampling and Purification of PBMCs

Thirty-four harbor seals (*Phoca vitulina*) were collected at Bic (48˚24'N, 68˚51'W) and at the coast of Métis-sur-Mer (48°41'N, 68°02'W), St. Lawrence Estuary in Quebec, Canada (Table 1, Figure 1). Pups were captured using a dip net and handled in an inflatable boat. Pups were weighed (+/- 0.5 kg), tagged, if necessary, and their gender determined. Handling was carried out within 20 min to avoid abandonment by the mother (Boulva *et al.*, 1979). Pups were captured from the 16th to 18th June 2014. Up to 10 mL of blood was taken from the extradural intravertebral vein into heparinized Vacutainer tubes (Becton-Dickinson, NJ, USA) and was kept cool until lymphocyte separation with Lympholyte-Mammal (Cedarlane, Burlington, Canada) within 6 h of blood sampling. The PBMCs were resuspended in completed RPMI-1640 (10 % FBS, 25 mM HEPES, 1 % Penicillin-Streptomycin; all from Sigma-Aldrich, Oakville, Canada) and kept at 4 °C overnight until the start of exposure. The research was approved by the Animal Care Committees of the Department of Fisheries and Oceans Canada (Protocol Number: 10-5).



II Figure 1. Map of the St. Lawrence River estuary. Two harbor seal (*Phoca vitulina*) haul-out sites were sampled on the shore of Métis-sur-Mer (star symbol; 48°40'N, 68°01'W) and around the Île du Bic in the Parc National du Bic (triangle symbol; 48°24'N, 68°48'W).

II Table 1. Details of harbor seal (*Phoca vitulina*) pups analyzed in this study.

Date of collection	Location (Canada)	n	Sex	Weight (kg)
16.06.2014	Bic	11	4 F - 7 M	14.5 - 27.5
17.06.2014	Métis	11	5 F - 6 M	11.5 - 25.5
18.06.2014	Bic	12	6 F - 6 M	16.0 - 28.5

4.2.2 Preparation of xenobiotics

Pharmaceuticals (all from Sigma-Aldrich, Oakville, Canada) were dissolved in DMSO. The final concentration of vehicle in the samples did not exceed 0.5 %. Four single compounds and four binary mixtures were tested. The full range of concentrations is described in Table 2.

The positive control, cadmium chloride ($CdCl_2$), was dissolved at 50 mg/L in sterile H_2O.

4.2.3 Lymphocyte proliferation assay

For the lymphoblastic proliferation assay 2.5×10^5 of cells were incubated with the pharmaceutical solution.

Change in lymphocyte proliferation after stimulation with the mitogen Concanavalin A (ConA; Sigma, Oakville, Canada) was measured as the incorporation of methyl-^{3}H-thymidine. After cells were incubated with pharmaceuticals and 5 µg/mL ConA for 48 h, 1 µCi of methyl-^{3}H-thymidine (PerkinElmer, Shelton, USA) was added and cells were incubated for an additional 18 h. 96-well plates were then kept at -20 ˚C until the cells were harvested onto a glass fiber filter (Tomtec Mach III Cell Harvester) and the amount of radioactive bases incorporated into the DNA was measured with a TriLux counter (Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer) and analyzed with the MicroBeta Windows Workstation (Version 4.50.09, PerkinElmer, Shelton, USA) software. The raw data were expressed as counts per minute (CPM) and later converted to percent proliferation relative to the negative control (NC, 100%).

II Table 2. Exposure concentration range (µg/L) of the individual pharmaceutical compounds and binary mixtures. Concentrations that induced significant reduction in proliferation are in bold. Dunnett's Multiple Comparison Test significance levels are indicated with asterisks (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

Name	**Compound**	**Concentration range** (µg/L)			
Concentration factor		**2**	**20**	**200**	**400**
17α-ethinyl estradiol	EE_2	250	***** 2,500**	***** 25,000**	***** 50,000**
Erythromycin	ERY	750	7,500	**** 75,000**	***** 150,000**
Naproxen	NAP	500	5,000	***** 50,000**	***** 100,000**
Carbamazepine	CZP	500	*** 5,000**	****** 50,000**	****** 100,000**
Name	**Compound**	**Concentration range** (µg/L)			
Concentration factor		**1**	**10**	**100**	**200**
EE_2 + ERY	EE_2	125	**1,250**	**12,500**	**25,000**
	ERY	375	****** 3,750**	****** 37,500**	****** 75,000**
EE_2 + NAP	EE_2	125	1,250	**12,500**	**25,000**
	NAP	250	2,500	***** 25,000**	***** 50,000**
NAP + ERY	NAP	250	2,500	**25,000**	**50,000**
	ERY	375	3,750	***** 37,500**	***** 75,000**
NAP + CZP	NAP	250	2,500	25,000	**50,000**
	CZP	250	2,500	25,000	***** 50,000**

Results obtained with ConA-stimulated harbor seal PBMCs were compared to results obtained with the LPS-stimulated 11B7501 harbor seal B lymphoma cell line (Chapter 1, Figure 5; Table 3). Raw data of the 11B7501 cell line were reutilized in this chapter to better illustrate the comparison.

4.2.4 Viability assay

Viability assays were set up in parallel with the lymphocyte proliferation assays to distinguish cytotoxicity from immunotoxicity. For each assay, 6.25×10^5 cells were incubated with the pharmaceutical product.

After 66 h, cell viability was evaluated adding 0.8 µg/mL propidium iodide (PI) (Sigma-Aldrich, Oakville, Canada) to the cell suspension. A FACSCalibur (Becton Dickinson, San Jose, CA, USA) with an air-cooled argon laser providing an excitation at 488 nm was used. For each sample 5,000 events were acquired at a fluorescence emission of 620 nm (FL3). The cell population was electronically gated in a FSC/SSC dot plot and the fluorescence frequency distribution histogram was obtained. The percentage of dead cells was determined using a marker to quantify the increase in fluorescence associated with cell death. Data collection and analysis were performed with the CellQuest Pro software (Version 4.0.1). The results were expressed in percentage of viable cells.

4.2.5 Statistical analyses

Differences between negative controls (NC, H_2O) and treated groups were evaluated by one-way ANOVA followed by a Dunnett's post hoc test for each compound ($p \leq 0.05$). Differences between male and female samples (gender x concentration of pharmaceutical) were analyzed by two-way ANOVA.

Data from the lymphoblastic proliferation assay were curve fitted using nonlinear regression analysis. Concentrations of pharmaceutical compound or binary mixture that cause 50 % inhibition of "B_{max}" (IC_{50}) were calculated. The IC_{50} data were normalized according to the Graph Pad Prism 6 equation "*log(inhibitor) vs. normalized response - Variable slope*":

$$Y=100/(1+10\text{^}((\text{Log}IC_{50}-X)*\text{HillSlope})),$$

where "Y" represents the percentage of proliferation, X represents the concentration of pharmaceutical product, "$\text{Log}IC_{50}$" represents the log of concentration of pharmaceutical product that gives a response half way between the maximum (B_{max}) and minimum (B_{min}) plateau of the sigmoidal curve and HillSlope describes the steepness of the family of curves.

To be able to compare individual with mixture toxicity, we transformed the concentrations of single substances and mixtures. We utilized the concentration factor (Table 2) to be able to present the dose response curves in one graph (Figure 3 A, C, E, G). The 1x concentration for the four compounds was EE_2 = 125 µg/L; ERY = 375 µg/L; NAP = 250 µg/L and CZP = 250 µg/L.

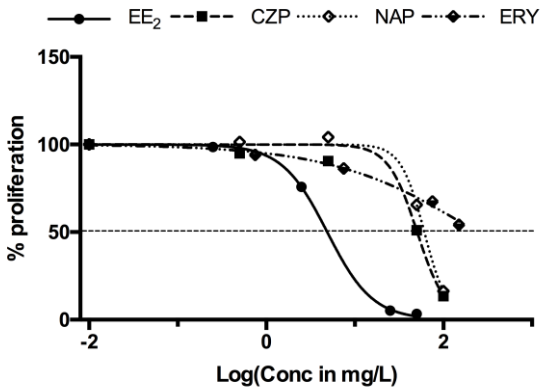
To further evaluate the mixtures, Pearson's correlation analysis was used to compare the measured to the calculated reduction in lymphocyte proliferation. The calculated reduction consisted of the sum of the response measured for its individual compounds.

The calculations were performed using GraphPad Prism 6 for Mac (GraphPad Software). The level of significance was set at $p \leq 0.05$.

4.3 Results

4.3.1 Relative toxicity of individual chemicals

EE_2, NAP, ERY and CZP all affected the proliferation of harbor seal PBMCs within the concentrations tested. The dose-response curves (Figure 2) and half maximal inhibitory concentration (IC_{50}) (Table 3) suggest that EE_2 is the most immunotoxic pharmaceutical tested, followed by CZP, NAP and ERY. In fact, the IC_{50} for EE_2 is one order of magnitude lower than that for CZP and NAP, and approximately 50 times lower than that for ERY.



II Figure 2. Lymphocyte proliferation of the harbor seal PBMCs to individual pharmaceuticals after 66 h exposure and ConA stimulation. The concentration of pharmaceutical product (mg/L) was log transformed before regression analysis. Results are expressed as Mean ± SEM.

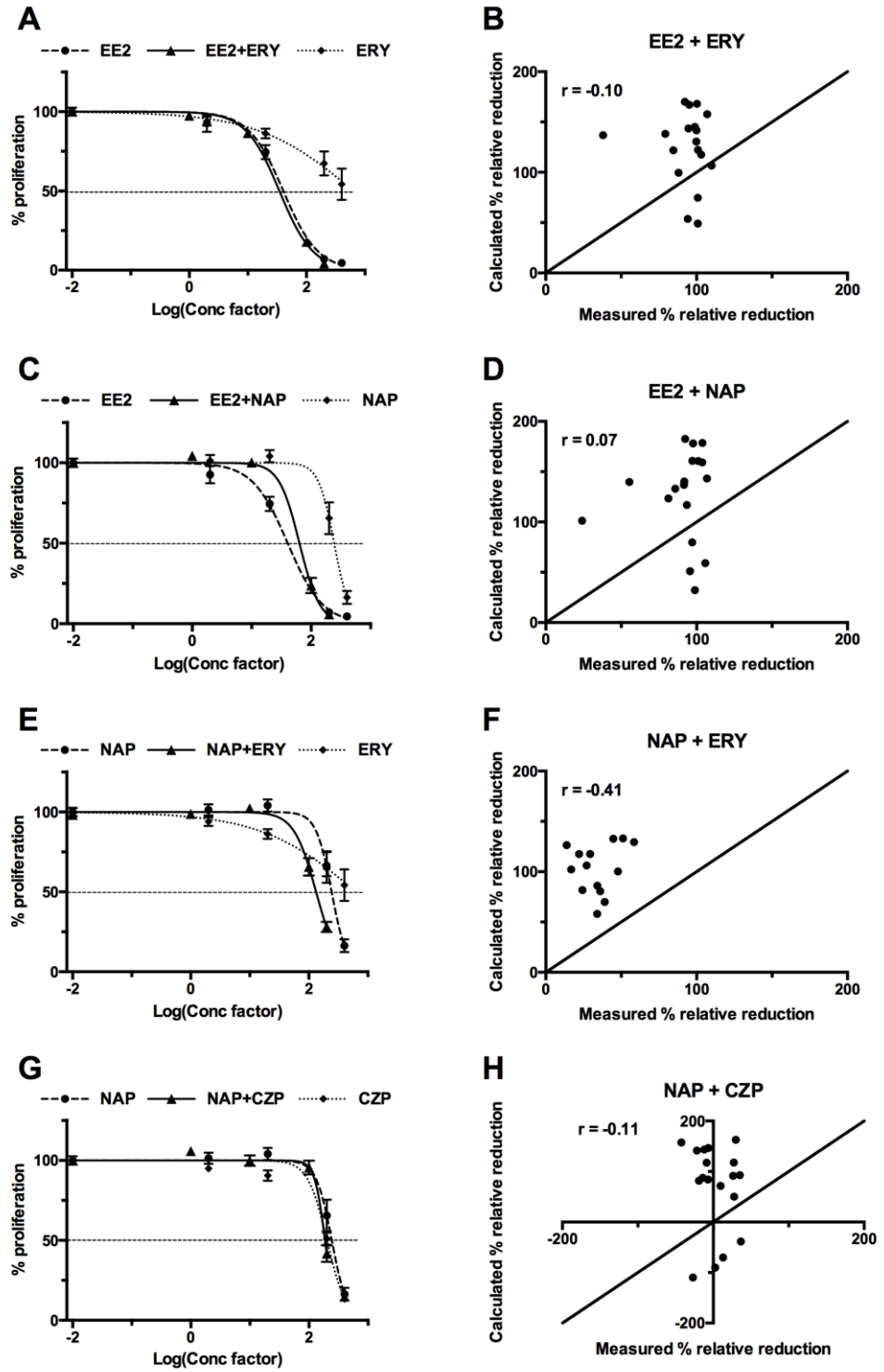
II Table 3. Half-maximal inhibitory concentration (IC_{50}) values (µg/L) for harbor seal PBMCs and the 11B7501 harbor seal B lymphoma cell line to individual pharmaceuticals after 66 h exposure and ConA (PBMC) or LPS (11B7501) stimulation. Raw data of the 11B7501 cell line were obtained from Kleinert *et al.*, 2016a, unpublished (Chapter 1) and reutilized in this table to better illustrate the comparison.

PBMCs					µg/L
	EE_2	**ERY**	**NAP**	**CZP**	
IC_{50}	5,044	237,713	60,870	50,525	
11B7501 B lymphoma cell line					µg/L
	EE_2	**ERY**	**NAP**	**CZP**	
IC_{50}	36,910	-	129,200	-	

4.3.2 Additive/Non-additive effects of mixtures

Whether binary mixtures of these four pharmaceuticals had additive or non-additive and synergistic or antagonistic effects was further examined (Figure 3, Table 3). ERY seemed to add little to the toxicity of the mixture of EE_2 and ERY, since the curves of the mixture and EE_2 (IC_{50} = 5,044 µg/L) overlap (Figure 3A, Table 3). The toxicity of the mixture of EE_2 and NAP was intermediate compared to the toxicity of the single substances, since the curve of the mixture is situated between the curves of EE_2 (IC_{50} = 5,044 µg/L) and NAP (IC_{50} = 60,870 µg/L) (Figure 3C; Table 3). A synergistic toxicity was observed for the mixture of NAP and ERY (Figure 3E; Figure 3F; Table 3), since individual compounds ERY (IC_{50} = 237,713 µg/L) and NAP (IC_{50} = 60,870 µg/L) were less toxic. Lastly, in the binary mixture of NAP and CZP, combination of both substances with similar toxicity did neither increase nor decrease the toxic effect (Figure 3G).

Pearson's correlation was used to assess whether mixtures had additive or non-additive effects compared to the effects of individual pharmaceutical compound alone (Figure 3 B, D, F, H). No significant correlations were detected in the four mixtures. These findings suggest that the individual compounds in mixtures had non-additive interactions with harbor seal PBMCs, and that the toxicity of a mixture could not be accurately predicted from that of its components.



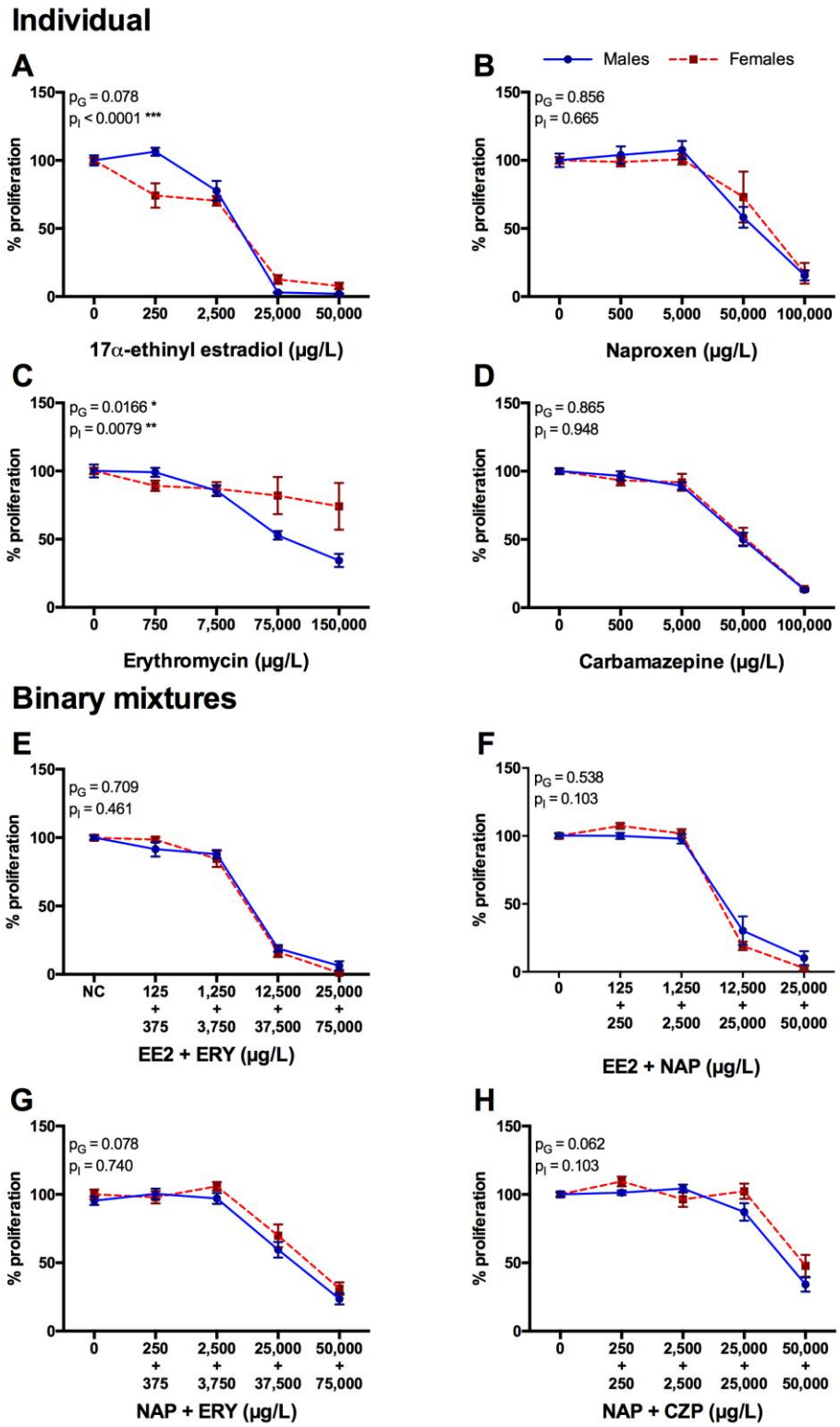
II Figure 3. Lymphocyte proliferation of the harbor seal PBMCs to individual pharmaceuticals and binary mixtures after 66 h exposure and ConA stimulation. Concentration factors of the pharmaceutical products were log transformed before regression analysis. Results are expressed as Mean ± SEM. The Pearson's correlation coefficient (r) is given for each mixture (B, D, F, H).

4.3.3 Influence of gender on toxicity

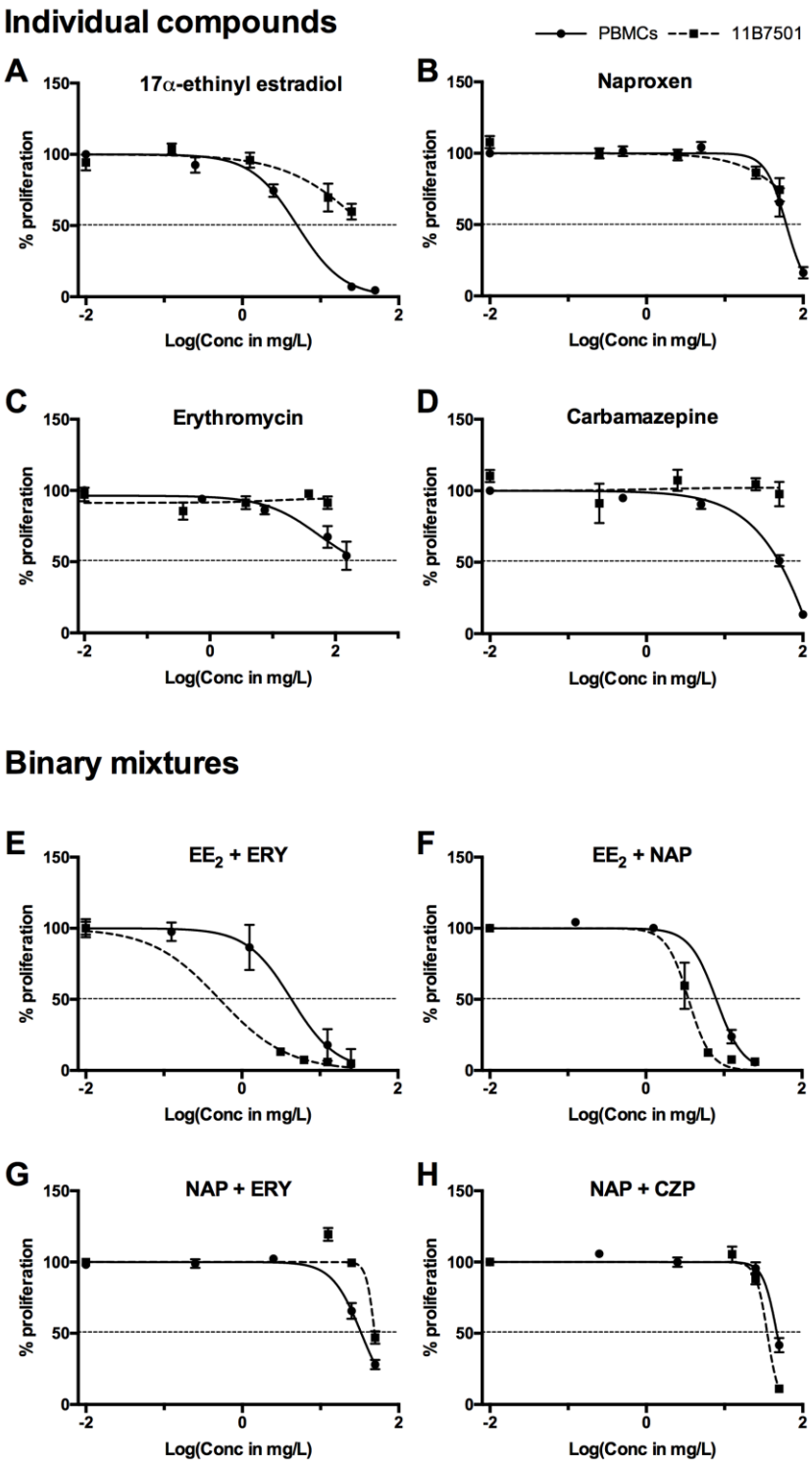
To further assess if there was a difference in toxicity associated with gender, we ran a two-way ANOVA. Firstly, it revealed a significant effect of exposure concentration in all individual compounds and binary mixtures, as expected (Figure 4 A-H, $p<0.0001$, all).

Secondly, the antibiotic erythromycin affected the two genders differently ($p=0.017$), with the PBMCs of female harbor seal pups being less sensitive to 75,000 and 150,000 µg/L ERY than PBMCs of male pups (Figure 4 C). The remaining individual compounds and binary mixtures did not influence PBMCs of female and male harbor seal pups differently.

An interaction of both main effects "exposure concentration" and "gender" was observed in ERY as well as EE_2 (Figure 4A and 4C). For EE_2, however, it did not result in a significant difference between genders ($p=0.078$).



II Figure 4. Lymphocyte proliferation of male and female harbor seal PBMCs exposed to individual and binary mixtures of pharmaceuticals after 66 h exposure and ConA stimulation. p_G describes the significance of the factor *gender* whereas p_I describes the significance of the *intercept* of both curves. Results are expressed as Mean ± SEM.



II Figure 5. Lymphocyte proliferation of harbor seal PBMCs and the 11B7501 harbor seal B lymphoma cell line to individual pharmaceuticals and binary mixtures after 66 h exposure and ConA (PBMC) or LPS (11B7501) stimulation. Raw data of the 11B7501 cell line were obtained from Kleinert *et al.*, 2016a, unpublished and reutilized in this figure to better illustrate the comparison. Results are expressed as Mean ± SEM.

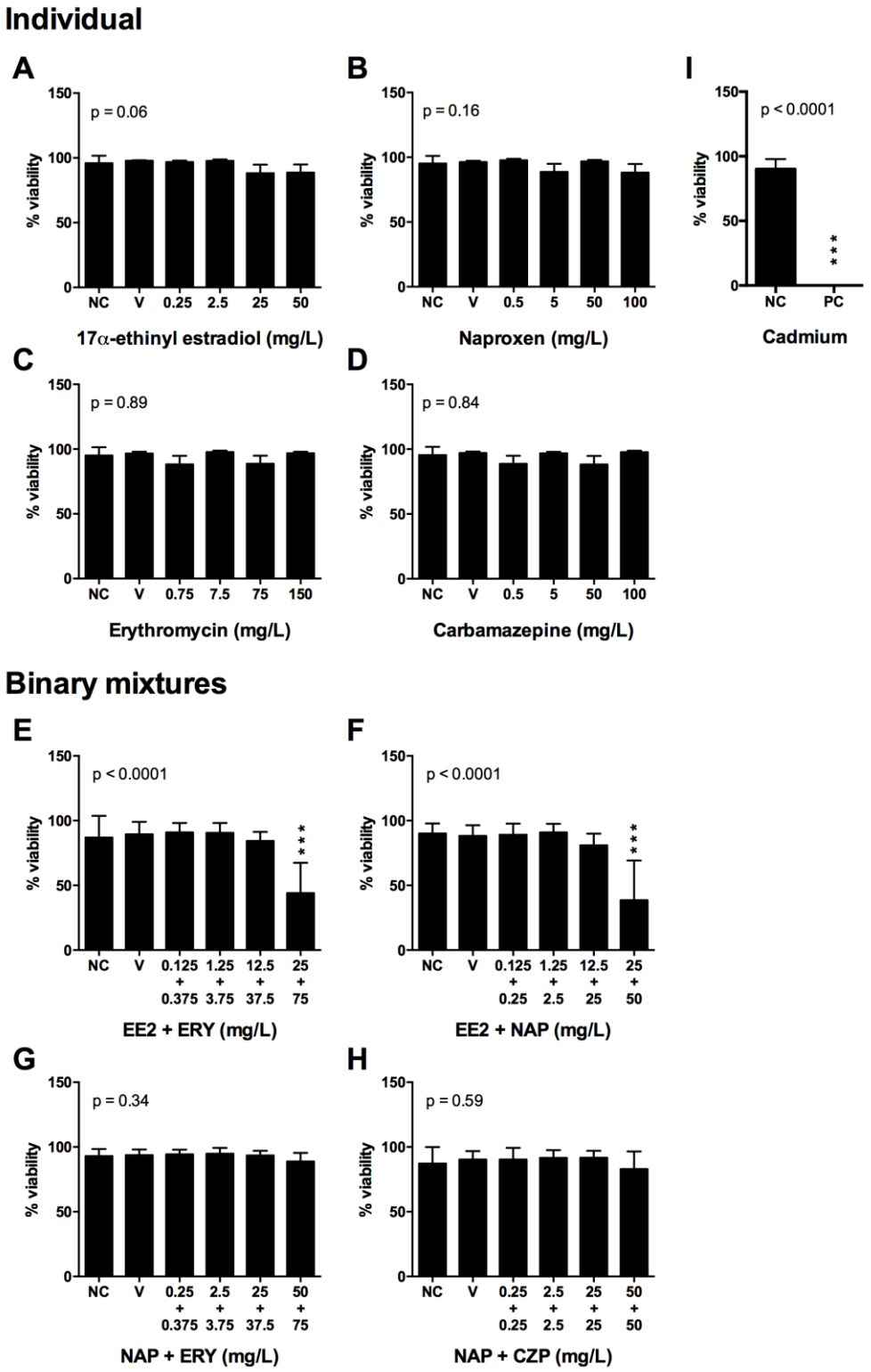
4.3.4 PBMCs vs. 11B7501 B lymphoma cell line

The sensitivity of harbor seal pup PBMCs (T lymphocytes) stimulated with Con A was also compared to harbor seal 11B7501 B lymphoma cell line stimulated with LPS (Figure 5, Table 3). For individual substances, the proliferation of T cells derived from PBMCs was more sensitive than the proliferation of B cells derived from a cell line (Figure 5 A-D; Table 3). The compounds ERY and CZP did not show any toxicity in the B lymphoma cell line in the concentration range tested, while the lymphocyte proliferation was significantly inhibited in ConA-stimulated T cells derived from PBMCs (IC_{50}= 237,713 µg/L; IC_{50}= 50,525 µg/L, respectively) (Figure 5 C-D).
For binary mixtures, the LPS-stimulated 11B7501 B lymphoma cell line was more sensitive in three out of four cases (Figure 5 E, F, H). Only the mixture NAP and ERY was affecting ConA-stimulated PBMCs more (IC_{50}=33,190 µg/L+ 49,785 µg/L) than the B lymphoma cell line (IC_{50}= 49,280 µg/L+73,920 µg/L) (Figure 5 G).

4.3.5 Viability

The viability of the harbor seal PBMCs was 89.3 ± 11.1 % in the NCs after 66 h of incubation for the lymphocyte proliferation assay. It was not significantly affected after incubation with any of the individual compounds (Figure 6 A-D).
A significant difference in viability was observed in the two binary mixtures containing EE_2 (Figure 6 E-F). Viability decreased to 44.1 ± 23.4 % for EE_2 and ERY at 25,000 µg/L + 75,000 µg/L and decreased to 38.5 ± 30.6 % for EE_2 and NAP at 25,000 µg/L + 50,000 µg/L ($p<0.0001$, both). The remaining mixtures did not show significant effects on viability (Figure 6 G-H).

Cadmium chloride ($CdCl_2$) at 50 mg/L decreased the viability of harbor seal PBMCs significantly ($p<0.0001$).



II Figure 6. Viability of PBMCs after 66 h of exposure to individual (A-D) and mixtures (E-H) of pharmaceutical compounds. Results are expressed as Mean ± SEM. Asterisks show a statistical difference from control cells (* $p \leq 0.001$).**

4.4 Discussion

Increasing awareness of the widespread occurrence of pharmaceuticals and personal care products (PPCPs) in the environment has generated interest in their potential health effects on wildlife populations. As pharmaceuticals are developed based on a specific biological activity and easily pass cell membranes by diffusion due to their non-polar structure (Khetan *et al.*, 2007), these chemicals can cause unintended side effects. Health effects in fish included feminization and intersex in male fish related to synthetic estrogen exposure at environmentally relevant concentrations (Jobling *et al.*, 1998, Tyler *et al.*, 1999), alterations in spawning patterns after NSAID (ibuprofen) exposure (Flippin *et al.*, 2007) and suppression of the immune system after antibiotics (tetracyclin) exposure (Grondel *et al.*, 1985, Wishkovsky *et al.*, 1987), just to name a few.

The immune system has been shown to be an interesting "biomarker" to assess, since an impact on the immune system can potentially lead to an altered host resistance and susceptibility to cancer or infectious diseases (Martineau *et al.*, 1994, Ross *et al.*, 1996c). The potential immunotoxicity of PPCPs has been previously reviewed in aquatic invertebrates (Matozzo, 2014). However, to date, nothing has been published about the potential immunotoxicity of PPCPs in aquatic top-predators (Daughton *et al.*, 2015). Among marine mammals, harbor seals are ideal model organisms for investigations on contaminant-induced immune alterations in the marine environment (Ross *et al.*, 1996c). Similar to many other marine mammals, harbor seals are prone to accumulate lipophilic anthropogenic compounds easily, since they are piscivorous, long-lived, maintain large adipose deposits and occupy high trophic levels in the food web (Ross *et al.*, 1996c). Furthermore, unlike other marine mammals, harbor seals are non-migratory, and frequently share their coastal habitats with human populations, exposing them to industrial centers, heavy marine traffic, as well as urban and agricultural runoff (Neale *et al.*, 2002).

Lymphocyte proliferation is the most frequently used method to assess marine mammal immunotoxicity (Desforges *et al.*, 2016) and considered a reliable measure of the T-cell-mediated immune response to viral pathogens (Neale *et al.*, 2002). While it represents an extreme reductionism relative to the complex nature of the immune system as a whole, it can provide insight into specific unintended effects of xenobiotics (Neale *et al.*, 2002). We observed clear suppressive effects on T cell mitogenesis related to PPCP exposure. Since most exposure

concentrations did not affect the viability of cells, the selected PPCPs directly impacted the T cell function. The relative ability to inhibit lymphocyte proliferation was strongest in EE_2, followed by CZP, NAP and ERY. Interestingly, harbor seal T lymphocytes were more sensitive to an inhibition of mitogen-induced proliferation by individual pharmaceuticals than the previously tested 11B7501 harbor seal B lymphoma cell line (Kleinert *et al*., 2016a, unpublished).

The slopes of the dose-response curves of the individual pharmaceuticals vary considerably. ERY was not highly toxic, resulting in a high IC_{50} value and a rather gradual curve. On the other hand, EE_2 exposure induced an IC_{50} value 50 times lower than that for ERY, with steeper slope. The slopes observed for NAP and CZP with nearly parallel curves and similar IC_{50} values were lying between EE_2 and ERY. Curves with a steep slope reflect xenobiotics that are harmless until a threshold concentration is exceeded. Beyond the threshold concentration these compounds induce toxic effects. If the threshold concentration is present in the environment, these compounds might cause significant harm to organisms. If the threshold concentration is not present, these compounds do not induce toxicity. Curves with a gradual slope, on the other hand, reflect xenobiotics that induce small, sublethal effects already at lower concentrations. These compounds might be a higher threat to safety in the environment than "threshold compounds". Since these compounds alter the physiological state of the immune system already at low concentrations, even if it is not a strong alteration, host resistance could already be impacted to some extent. Also, these compounds should be prioritized for analysis in mixture toxicity to assess possible potentiation of effects.

Next to assessing the immunotoxic potential of individual PPCPs, we chose to assess the impact of binary mixtures of these substances. We found that by simply adding the toxicity of individual PPCPs, the reduction of lymphocyte proliferation exposed to a mixture would generally be overestimated. This finding is in contrast to a previous study evaluating the impact of the same pharmaceutical mixtures on the 11B7501 harbor seal B lymphoma cell line. Predicted toxicity for mixtures on the seal cell line underestimated the inhibitory effects on lymphocyte proliferation since mixture effects were synergistic (Kleinert *et al*., 2016a, unpublished). Synergistic tendencies were also found in two studies on the Rainbow trout (*Oncorhynchus mykiss*) RTL-W1 hepatocyte and RTG-2 gonadal cell lines evaluating cytotoxicity. Dissimilar

mixtures of pharmaceuticals induced higher than additive toxicity in both cell lines (Fernandez *et al.*, 2013, Schnell *et al.*, 2009).

It would have been interesting to compare results obtained with harbor seal pup PBMCs with adult free-ranging PBMCs, which was unfortunately not possible in the course of our study. Previously, different proliferative responses were observed in pup and adult lymphocytes after exposure to butyltins, with pup lymphocytes being stimulated after exposure to 200 nM tributyltin, while adult harbor seal lymphocytes were suppressed at the same concentration (Frouin *et al.*, 2008).

Gender could be a factor influencing the immunotoxicity of PPCPs, though only ERY showed an effect on lymphocyte proliferation that was significantly higher in male than in female pups. Previously, female seals have been shown to be more sensitive to contaminant exposure than male seals (Brousseau *et al.*, 1999). In the case of PPCPs, this tendency might however be inversed, as our initial results indicate, since male pups seemed to be slightly more sensitive.

Since pharmaceuticals occur in the aquatic environment in concentrations from ng/L up to µg/L range, it is rather rare that *in vitro* methods with short exposure times are sensitive and specific enough to measure effects at the organismal level. With only 66 h of exposure time, we therefore expected effects in the mg/L range. The acute immunotoxic potential of pharmaceuticals is inferior to classic contaminants, since there are intended for short-term application in a biological system. However, pharmaceuticals are still compounds of emerging concern, because consumption is increasing, regulation is lacking and sewage treatment plants (STPs) are not equipped to remove them before they are introduced into the environment. Organisms in the environment are therefore chronically exposed to pharmaceuticals amongst other xenobiotics.

It is difficult to draw causal relationships from circumstantial evidence, however the weight of evidence shows that immunotoxicity leads to a decrease in host resistance to pathogens (Luster *et al.*, 1993). Previous research on extensive testing batteries concluded that most immune function-host resistance relationships followed linear rather than threshold like models (Luster *et al.*, 1993). To better understand the relationship between contaminants, immune function, and outbreaks of disease in marine mammals, *in vitro* laboratory experimentation and work with captive and free ranging animals need to be combined (Ross *et al.*, 1996c). Furthermore, for individual studies it is beneficial to combine more than one immune test to determine immuno-

toxicity (Luster *et al.*, 1993). The proliferative response to LPS was found to be a poor indicator for host resistance changes (Luster *et al.*, 1993). When combined with other methods assessing immunotoxicity specificity and sensitivity ranged between 56-90 % (mean: 71.2 ± 10.6 %). T cell mitogens on the other hand had a range of 62-92 % (mean: 76.7 ± 8.5%). Ideally, LPS mitogen response or T cell mitogens should be combined with cell surface markers, since this lead to a sensitivity and specificity of over 90 % (Luster *et al.*, 1992).

4.5 **Conclusion**

In conclusion, our findings suggest a potential for pharmaceuticals to act as immunosuppressants on harbor seal T lymphocyte function, which could lead to an impaired host resistance against viral pathogens *in vivo*. To our knowledge, this is the first study to investigate pharmaceutical exposure in harbor seals and directly demonstrate immunotoxic effects of model pollutants in the aquatic environment on functional responses of T lymphocytes in a marine mammal species. Future work should include a wider range of pharmaceutical compounds and mixtures and further highlight confounding factors like age and gender.

Section 5.

Chapter 3 – Immunotoxicity of Pharmaceuticals on Gene Expression

5 CHAPTER 3

IMMUNOTOXICITY OF PHARMACEUTICALS ON GENE EXPRESSION

5.1 Introduction

Harbor seals are exposed to various anthropogenic stressors in the environment due to their coastal habitat, e.g. chemical pollution, litter (plastics and nanoparticles), noise, and climate change (Harwood, 2001, Lehnert *et al.*, 2016, Weijs *et al.*, 2008). Since exposure to persistent organic pollutants and metals has been shown to suppress the seal's immune system (Das *et al.*, 2008, Ross *et al.*, 1996c), it is important to understand the relative contributions of other stressors to immunosuppression as well.

Pharmaceuticals are a compound class that has been only recently analyzed for their immunosuppressive potential in marine mammal leukocytes (Kleinert *et al.*, 2017, Kleinert *et al.*, 2013). The synthetic estrogen 17α-ethinyl estradiol (EE_2), which is the active compound of the contraceptive pill, has been the focus of this study. Firstly, it is an environmental xenobiotic and frequently found in municipal effluents and surface waters (Allinson *et al.*, 2010). Secondly, EE_2 has previously affected lymphocyte proliferation and the cell cycle of the harbor seal 11B7501 B lymphoma cell line and harbor seal peripheral blood mononuclear cells (Kleinert et al., 2016a, unpublished; Kleinert et al., 2016b, unpublished). Lastly, B lymphocytes and lymphomas are regulated by estrogens through estrogen receptors (ERα and ERß) in mammals (Grimaldi *et al.*, 2002, Medina *et al.*, 2000). An active ERß receptor acts as an anti-proliferative and pro-apoptotic stimulus in non-Hodgkin lymphoma in humans (Yakimchuk *et al.*, 2011).

The model used in this study was the harbor seal 11B7501 B lymphoma cell line. This lymphoma cell line is an ideal proxy for immunocompetence assessments in marine mammals since confounding factors like physiological state and inflammation of an individual are of no concern.

New molecular biomarkers have recently been developed to identify early biological effects using minimally invasive blood samples (Lehnert *et al.*, 2014, Müller *et al.*, 2013, Weirup *et al.*,

2013). Since pharmaceuticals are designed to have a low acute toxicity, these potentially more sensitive methods might be more relevant in assessing immunotoxicity for this class of compounds and help infer underlying mechanisms of toxic action.

The objectives of this study were to determine the cytotoxicity of EE_2 and gene expression profiles in a harbor seal B lymphoma cell line.

5.2 Material and Methods

The harbor seal 11B7501 B lymphoma cell line (CRL-1940 purchased from ATCC, Manassas, VA) was maintained as previously described (Frouin *et al.*, 2010a). 17α-ethinyl estradiol (EE_2) was dissolved in dimethyl sulfoxide (DMSO) (both Sigma-Aldrich, Oakville, Canada). The final concentration of DMSO in the samples did not exceed 0.1%.

For the *in vitro* exposures, 1×10^6 of cells were incubated with EE_2 for 24 h. EE_2 concentrations were 0, 1, 10, 100, 1 000, 10 000, or 25 000 µg/L. In a parallel set of experiments the cell line was exposed to the same concentrations of EE_2 in the presence of 5 µg/mL of the mitogen lipopolysaccharide (LPS). LPS activated B lymphocytes non-specifically (induced proliferation) and was meant to simulate the cell activity for proliferation as if under pathogen exposure.

RNA was extracted using the Aurum total RNA kit (Bio-Rad, Mississauga, ON). Nanodrop-normalized (A260 nm) levels of total RNA were reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON). cDNA were used for quantitative polymerase chain reaction (qPCR) using Ssofast Evagreen supermix (Bio-Rad, Mississauga, ON). qPCR was initiated with 95 ˚C for 30 s, followed by 40 cycles with denaturation at 95 ˚C for 5 s, and the primer specific annealing temperature (62 ˚C) for 15 s. Melting curves were created by denaturation at 95 ˚C followed by 61 steps during which the temperature was increasing steadily for 0.5 ˚C every 5 s, starting at 65 ˚C and ending at 95 ˚C, to exclude the measurement of non-specific PCR products and primer dimers and to determine true amplification. Results were analyzed using the comparative Ct method. Primers are shown in Table 1.

Three reference genes (ßACT, ß2M, YWHAZ) have been evaluated and compared as internal standards, and ß2M was chosen to normalize mRNA transcription levels of the genes of interest, since its expression was most stable when compared to the levels of total RNA. Genes of cells

exposed to EE_2 only were normalized with the ß2M 0 µg/L EE_2 negative control, whereas genes of cells exposed to EE_2 and LPS were normalized with the ß2M 0 µg/L EE_2+LPS negative control. It has to be noted that beta actin (ßACT) was not an ideal candidate as a reference gene in the 11B7501 cell line. ßACT is a non-muscle cytoskeletal actin isoform that is involved in cell motility, structure and integrity. It was significantly downregulated at 25,000 µg/L EE_2 and at 25,000 µg/L EE_2+LPS (Table 2). Although ßACT is a typical reference gene due to its function in the cytoskeleton, its application in studies on lymphocytes is not recommendable. For instance, a previous study on mouse lymphocytes has also found ßACT not to be a suitable reference gene due to its extensive variability in expression (Albershardt *et al.*, 2012).

Viability assays were set up in parallel to observe if EE_2 concentrations were cytotoxic to the 11B7501 B lymphoma cell line. After 24 h, viability of cells was evaluated by adding 0.8 µg/mL propidium iodide (PI) (Sigma-Aldrich, Oakville, Canada) to the cell suspension. A FACSCalibur (Becton Dickinson, San Jose, CA, USA) with an air-cooled argon laser providing an excitation at 488 nm was used. For each sample 5,000 events were acquired at a fluorescence emission of 620 nm (FL3). The cell population was electronically gated in a FSC/SSC dot plot and the fluorescence frequency distribution histogram was obtained. The percentage of dead cells was determined as the proportion of cells with an elevated fluorescence using a marker. Data collection and analysis were performed with the CellQuest Pro software (Version 4.0.1). The results were expressed in percentage of viable cells.

Differences between controls and treated groups were evaluated by one-way and two-way ANOVA followed by Dunnett's post hoc test. The calculations were performed using GraphPad Prism 6 for Mac (GraphPad Software). The level of significance was set at $p \leq 0.05$.

III Table 1. Primers used for qPCR analysis. All primers are listed from 5' to 3'.

Gene	Description	Forward (F) & Reverse (R) Primers
Housekeeping Genes		
ßACT	Involved in cell motility, structure and integrity	F' GAC TAC CTC ATG AAG ATC CT R' TCT TCT CCA GGG AGG AGG
ß2M	Component of MHC class I molecules	F' CTA CGT GTC AGG GTT CCA T R' TGC TTT ACA CGG CAG CTA
YWHAZ	Regulator of apoptotic pathways	F' AGA CGG AAG GTG CTG AGA R' ATC ACC AGC AGC AAC TTC
Genes of Interest		
AHR	Regulator of cytochrome P450	F' ATA CAG AGT TGG ACC GTT TG R' AAG AAG CTC TTG GCT CTT A
CD9	Regulator of cell development, activation, growth and motility	F' TCT TTG GCT TCC TCT TGG T R' TTG GAC TTC AGC TTG TTG TA
HSP70	Aids with protein folding under heat or chemical stress	F' GCA ACG TGC TCA TCT TTG A R' AGC CTG TTG TCA AAG TCC T
IFNγ	Cytokine; activates macrophages; induces MHC class II expression	F' CAA GGC GAT AAA TGA ACT CA R' CGG CCT CGA AAC AGA TTC
LYN1	Key enzyme in regulation of cell activation in hematopoietic cells	F' CAA GGG AAG GTG CCA AAT T R' GAC CAT ACA TCA GAC TTA ATC G
MAPKK3	Phosphorylates mitogen-activated protein kinase (MAPK)	F' TTG GTG GAT TCT GTA GCC A R' AAG CCC ACA CAT CAG ACT T
SLAM	Surface receptor of activated T and B cells; enhances proliferation and IFNγ production	F' CAT GAC CCT GGA GGA GAA R' CAA GCT GCA GTT CCC ATT
TGFß1	Cytokine involved in cell growth, proliferation, differentiation and apoptosis	F' ACA CCA ACT ACT GCT TCA G R' GCA GAA GTT GGC GTG GTA

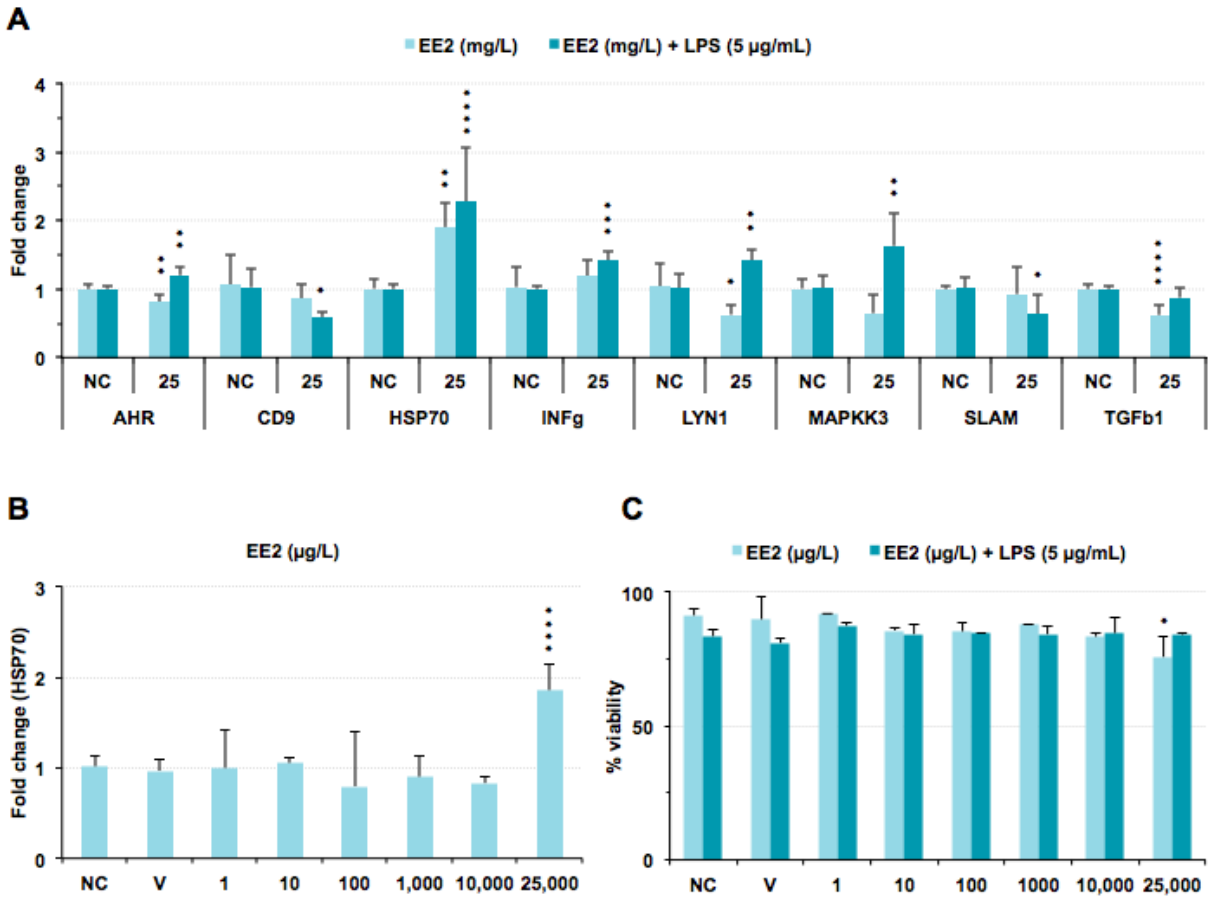
Abbreviations: ßACT = beta actin; ß2M = beta-2 microglobulin; YWHAZ = 14-3-3 protein zeta/delta; AHR = aryl hydrocarbon receptor; CD9 = Cluster of differentiation 9; HSP70 = 70 kDa heat shock protein; IFNγ = interferon gamma; LYN1 = Lck/Yes novel tyrosine kinase; MAPKK3 = mitogen-activated protein kinase kinase; SLAM = signaling lymphocytic activation molecule; TGFß1 = transforming growth factor beta 1.

5.3 Results and Discussion

A range of eight immune-relevant (CD9, IFNγ, LYN1, MAPKK3, SLAM, TGFß1), pollutant-associated (AHR) and stress (HSP70) biomarkers as well as three reference genes (ß2M, YWHAZ and ßACT) were selected to analyze differential gene expression in the harbor seal 11B7501 B lymphoma cell line exposed to EE_2. We limited our choice of genes to sequences that have already been validated in the harbor seal.

To obtain an overview of potential effects on the mRNA expression level of the chosen genes, we analyzed the control samples and the highest exposure concentration of EE_2 (25,000 µg/L) for the genes of interest (Figure 1A). The mRNA expression of all but one (ß2M) gene was impacted at 25 000 µg/L EE_2. Genes at this concentration were both up- and downregulated. Lymphocytes exposed to EE_2 without stimulation from LPS downregulated four out of five genes (Table 2). Lymphocytes exposed to EE_2 and the mitogen LPS were more likely to have changes in gene expression, and genes were mainly upregulated (6 of 9) (Table 2).

The HSP70 protects proteins from stress by aiding protein folding. HSP70 mRNA was upregulated two-fold compared to controls upon exposure to EE_2 alone as well as EE_2 and LPS (Table 2). Since HSP70 showed the most marked change in gene expression of all genes tested, we further evaluated the remaining exposure concentrations. The analysis revealed that HSP70 was induced only at the highest concentration (Figure 1B). Previous studies in harbor seal blood demonstrated negative (cadmium, Cd) and positive (lead, Pb) correlations between trace metal concentrations in blood and HSP70 expression (Lehnert *et al.*, 2016). These trends are in accordance with previous work that demonstrated that xenobiotic exposure could lead to immunosuppression or acute and chronic inflammatory processes that could cause hypersensitivities or autoimmune diseases (Kakuschke *et al.*, 2007). When assessing HSP70 in blood of free-ranging animals it has to be taken into account that capture and sampling stress might alter the physiological levels of HSP70 in these animals. HSP70 and cortisol were correlated in adult seals, and habituation (i.e. decreasing levels of HSP70 over time) was observed in rehabilitation processes where seals have been handled frequently (Fonfara *et al.*, 2008, Lehnert *et al.*, 2014, Lehnert *et al.*, 2016, Weirup *et al.*, 2013).



III Figure 1. (A) Fold change in gene expression after 24 h exposure to 0 (NC) and 25 mg/L EE_2 as measured by qPCR. A fold change of 1 indicated no change in comparison to the reference gene ß2M. (B) Dose-response gene expression changes of HSP70 mRNA after 24 h exposure to EE_2. (C) Viability of the 11B7501 B lymphoma cell line after 24 h exposure to EE_2 and EE_2 + LPS. Results are expressed as Mean ± SD. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). *Abbreviations:* EE_2=17α-ethinyl estradiol; LPS=lipopolysaccharide; ßACT=beta actin; YWHAZ=14-3-3 protein zeta/delta; AHR=aryl hydrocarbon receptor; CD9=Cluster of differentiation 9; HSP70=70 kDa heat shock protein; IFNγ=interferon gamma; LYN1=Lck/Yes novel tyrosine kinase; MAPKK3=mitogen-activated protein kinase kinase; SLAM=signaling lymphocytic activation molecule; TGFß1=transforming growth factor beta 1.

Interferon gamma (IFNγ) and signaling lymphocytic activation molecule (SLAM) are associated with susceptibility to mammalian infectious diseases (Pacheco *et al.*, 2008). IFNγ is a cytokine that activates macrophages and induces class II major histocompatibility complex (MHC II) expression. In our study, IFNγ expression was upregulated after exposure to 25 000 µg/L EE_2 + LPS, but not when the mitogen LPS was not present (Figure 1A, Table 2). Since the +LPS negative control was identical to the -LPS negative control, this upregulation can be attributed to a combined effect of EE_2 with LPS.

III Table 2. Summary of significant gene expression changes in the 11B7501 B lymphoma cell line. Up- or downregulation is described as the percentage of expression relative to the negative controls of ß2M. The ß2M negative control (-EE_2/-LPS) was used to normalize genes exposed to EE_2 only, whereas the ß2M negative control (-EE_2/+LPS) was used to normalize genes exposed to EE_2 and LPS.

Genes	25,000 µg/L EE_2	25,000 µg/L EE_2 + 5 µg/mL LPS
AHR	↓ □□ ± 12□□	↑ □20 ± 13□□
CD9		↓ 59□± 7 □
HSP70	↑ □91□± 36 □	↑ 228 ± 80□□
IFNγ		↑ 141 ±□13 □
LYN1	↓ 63 ± 14□□	↑ 142 ± 17□□
MAPKK3		↑ 163□± 48 □
SLAM		↓ 63 ±□29 □
TGFß1	↓ 62□± 15 □	
YWHAZ		↑ □21 ±□23 □
ßACT	↓ 48 ± 8□□	↓ 68□± 9 □

Abbreviations: EE_2 = 17α-ethinyl estradiol; LPS = lipopolysaccharide; ßACT = beta actin; YWHAZ = 14-3-3 protein zeta/delta; AHR = aryl hydrocarbon receptor; CD9 = Cluster of differentiation 9; HSP70 = 70 kDa heat shock protein; IFNγ = interferon gamma; LYN1 = Lck/Yes novel tyrosine kinase; MAPKK3 = mitogen-activated protein kinase kinase; SLAM = signaling lymphocytic activation molecule; TGFß1 = transforming growth factor beta 1.

SLAM is a surface receptor on activated B and T lymphocytes. In our study, its expression was not affected by 25,000 µg/L EE_2 alone, but it was one of the few genes that was significantly downregulated when additionally exposed to LPS. It has been shown to enhance IFNγ production, and is the primary cellular receptor for *Morbillivirus* (McCarthy *et al.*, 2011). Interestingly, SLAM and IFNγ mRNA levels showed inverse trends in regulation after exposure to EE_2 and LPS. While the exposure to the EE_2 or LPS alone did not change the mRNA expression, the additional stimulus and stress of a potential pathogen (LPS) impacted the B

lymphoma cell line. The increase in IFNγ can therefore be attributed to the presence of LPS, and not due to an increased production due to SLAM.

Tyrosine-protein kinase LYN1 is involved in the regulation of cell activation of hematopoietic cells. LYN1 expression was upregulated after exposure to 25,000 µg/L EE_2 + LPS, but significantly downregulated when LPS was not present (Figure 1A, Table 2). These results are in accordance with previous work demonstrating an upregulation of LYN in murine macrophages after LPS as well as IFNγ stimulation (Boulet *et al.*, 1992).

Mitogen-activated protein kinase kinase 3 (MAPKK3) activates MAPK3, a protein involved in the regulation of cellular processes such as proliferation and differentiation in lymphocytes (Neale *et al.*, 2004). MAPKK3 mRNA was upregulated in response to 25,000 µg/L EE_2 + LPS exposure, but not when the mitogen LPS was not present (Figure 1A, Table 2). A change in the MAP kinase pathway could impact both B cell receptor or T cell receptor signaling and T cell development (Abbas *et al.*, 2003, Alberolaila *et al.*, 1995, Li *et al.*, 1996).

The observed downregulation of the transmembrane protein CD9 at 25,000 µg/L EE_2 + LPS is potentially related to the inactivation of histone deacetylases by LPS (Jin *et al.*, 2013).

TGFß1, a cytokine involved in cell growth, proliferation, differentiation and apoptosis, is the only gene affected by EE_2 alone, but not EE_2 + LPS.

As the differential gene expression patterns revealed (Figure 1A), it is crucial to test the immunotoxicity of a compound with and without stimulation from a potential pathogen like LPS, since immunotoxicity of a compound can vary quite considerably in the two exposure scenarios.

Viability of the harbor seal 11B7501 B lymphoma cell line after 24 h was 91 ± 3 % in the negative control and 83 ± 2 % in the negative control of cells exposed with LPS (Figure 1C). The cell line was significantly impacted only in one exposure at 25,000 µg/L EE_2 without LPS reducing it to 76 ± 8 %. Changes in gene expression at this concentration can therefore have occurred also due to a cytotoxic reaction rather than an immunotoxic effect as in the non-cytotoxic exposure at 25,000 µg/L EE_2 + LPS that directly impacted the B cell function of the 11B7501 B lymphoma cell line.

It is problematic to conclude environmentally relevant effects of EE_2 on gene expression of harbor seal lymphocytes in this study, since the doses of EE_2 were quite high. Furthermore, it

should be investigated to what extent hormone levels in FBS might have influenced the bioavailability of EE_2 to the cells, by comparing with charcoal stripped FBS.

Quantitative PCR has rarely been utilized in toxicity testing in the marine mammal immune system (Lehnert *et al.*, 2016), while the method is more common in other species. To our knowledge this is the second study after Lehnert *et al*., 2016 that has used this method to assess the toxic mechanisms of action of gene expression in marine mammal immune cells. To date, only few mRNA sequences are known for pinnipeds in comparison to e.g. rodent sequences. It is therefore important to continue the effort to sequence the gene expression transcript and continue using marine mammal lymphocyte cell lines or blood samples from free-ranging animals to further the knowledge of immunotoxic action under contaminant load in marine mammals.

In conclusion, the observed effect of EE_2 depends on the activation status of the lymphocyte, therefore pinpointing complexity of such studies. Indeed, the present results show that two genes were expressed differently with or without LPS. Two genes reacted similarly with or without LPS. One gene was downregulated with EE_2 alone, while two genes were downregulated and three upregulated with EE_2+LPS. We estimate that gene expression analysis is a useful tool in marine mammal immunotoxicological research that should be further developed and used.

Section 6.

General Discussion and Perspectives

6 GENERAL DISCUSSION AND PERSPECTIVES

Anthropogenic pollution and its effect on human and ecosystem health is one of the major problems we are facing nowadays. This thesis project tried to elucidate a small proportion of this problem. The main objectives of this work were to i) test the acute toxicity of active pharmaceutical ingredients in harbor seal lymphocytes, ii) determine the relative toxicity of API mixtures, iii) compare different *in vitro* models and their sensitivity to APIs, and iv) determine sensitivity of different biomarkers on the cellular and gene expression level.

6.1 Main Findings

The acute toxicity of the selected pharmaceuticals individually was in the mg/L range for the 11B7501 B lymphoma cell line and harbor seal T lymphocytes. Not all of the selected compounds showed an effect individually.

The toxicity of binary mixtures was tested and non-additive effects were observed. The mixtures had synergistic effects in the harbor seal B lymphoma cell line and mixed effects on lymphocyte proliferation in harbor seal T lymphocytes.

Harbor seal blood T lymphocytes were more sensitive to changes in lymphocyte proliferation for individual pharmaceuticals generally than the 11B7501 cell line, but the cell line was inhibited to a greater extent than the T lymphocytes in three out of four mixtures.

There are first indications that male harbor seal lymphocytes might be more sensitive to pharmaceuticals and pharmaceutical mixtures than female lymphocytes.

In the case of 17α-ethinyl estradiol, the most toxic pharmaceutical in our list of selected APIs at the concentrations tested, we wanted to verify if biomarkers on the gene expression level might be more sensitive than lymphocyte proliferation. We observed an unspecific, receptor-unrelated toxicity with an increase in 70 kDa heat shock protein at the same concentration that also decreased the lymphocyte proliferation in the cell line. In case of EE_2, gene expression analysis was equally sensitive than biomarkers on the cellular level.

6.2 Choice of Biomarkers

There is a multitude of biomarkers that can be assessed to observe changes in the immune system of organisms. Desforges *et al*., 2016 summarized all immune-biomarkers that have been tested in marine mammals so far. Biomarkers included lymphoid tissue histopathology, circulating immune cells and hematology, lymphocyte proliferation, phagocytosis and respiratory burst, NK cell activity, antibody production and cytokines.

In this work, we chose to assess cellular function after exposure to pharmaceuticals with the lymphocyte proliferation assay for B and T lymphocytes, and furthermore assess the changes in the cell cycle and apoptotic events in the B lymphoma cell line. To distinguish changes in cell behavior due to cytotoxicity, we also chose to assess the viability of the cells in both cell types.

To explain mechanisms of action behind these changes in cellular function, it would have been ideal to set up mRNA microarrays in parallel to get a comprehensive overview of the changes in gene expression that accompany changes at the cellular level, and to discover patterns of genes that act together during an exposure to immunotoxic doses of pharmaceuticals. Whole genome scale transcriptomics are a promising approach for the health assessment in marine mammals (Mancia *et al.*, 2012). Since microarrays can generate a lot of data they are a very time effective tool that should be further developed in future research. Currently, there are no commercially available marine mammal microarrays, but assays have been developed for bottlenose dolphin (*Tursiops truncatus*) immune and stress genes (Mancia *et al.*, 2007). Alternatively, commercially available canine microarrays have been successfully customized for the use in California seal lions (*Zalophus californianus*) (Mancia *et al.*, 2012).

In any case, microarray data are usually controlled by assessing a selection of genes with quantitative PCR (qPCR) for comparison. When microarrays are not feasible, qPCR is a very adequate alternative to obtain limited but similar results. Due to a pre-selection of genes, only specific mechanisms of action can be targeted by qPCR, and therefore pre-selection has to be done careful. Published mRNA sequences for marine mammals are relatively limited to date. However, homologies in conserved sequences of more widely studied related species in the suborder caniformia (i.e. dog) can be used to design primers and determine unpublished mRNA sequences.

6.3 Choice of Compounds and Exposure Concentration

The ten pharmaceutical compounds of this work have been chosen due to their occurrence in wastewater and fish and demonstrated immunotoxic potential. Of course, since there are thousands of different pharmaceuticals in use to date, there are many more interesting compound candidates that could have been analyzed.

Antidepressants are some of the most prescribed drugs in the western world. In the U.S.A. one in ten people are being prescribed some form of antidepressant. Selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) are very common, and today's gold standards for these medications are escitalopram (brand names: Lexapro and Cipralex) and venlafaxine (brand names: Effexor or Lanvexin). Serotonin (5-hydroxytryptamine) is a neurotransmitter, which has important functions that exceed the nervous system. It is involved in movements of the digestive tract, bone metabolism, organ development, cardiovascular growth and placental function and development. An interruption of the serotonin balance could therefore have a variety of effects in the organism that could impact the fitness and health. In our study we examined the SSRI paroxetine (brand names: Paxil and Seroxat). Much more research has however been conducted for the SSRI fluoxetine (brand names: Prozac and Sarafem). In future research, it would be interesting to focus on the new generation chemicals like venlafaxine and determine their mechanisms of action in immune cells as well as different organs in marine mammals.

Another drug class that we have not assessed in the course of this work are statins, a lipid-lowering agent. The number one drug that is sold is the statin atorvastatin. During the course of this work, we analyzed the lipid-lowering fibrate gemfibrozil. While gemfibrozil did not display an immunotoxic effect on lymphocyte proliferation in the concentration ranges tested, these classes of drugs should remain a concern. Lipid-lowering drugs and their effects on marine mammals should be further investigated in the future beyond the field of immunotoxicity. Marine mammals rely on their extensive blubber layer for thermal insulation and prevention of hypothermia. Furthermore, statins and fibrates could potentially release liposoluble contaminants accumulated in the blubber layer and make them bioavailable again. Individuals exposed to lipid-lowering agents could therefore potentially have a significant fitness disadvantage.

Concentrations tested in our *in vitro* assays are in the µg/L to mg/L range. Even though pharmaceuticals have never been measured in marine mammal serum, we doubt that these concentrations are environmentally relevant exposure concentrations for marine mammal leukocytes. A significant focus of chemical analyses has been the detection of pharmaceuticals in effluents. Fewer studies have analyzed pharmaceuticals in surface water and even less in sediment (see Literature review). Not all accumulation studies in fish have used environmentally relevant exposure scenarios, and therefore it is difficult to estimate realistic exposure concentration of pharmaceuticals of different immune components in marine mammals. Recently, personal care product accumulation has been analyzed for the first time in marine mammals and revealed that coastal species are indeed exposed to UV filters found in sunscreen or to triclosan, an antimicrobial additive. But in general, both accumulation studies as well as immunotoxicity studies are still largely lacking for marine mammals.

6.4 Acute vs. Chronic Immunotoxicity

The thesis work was based on the determination of acute toxicity and short-term exposure of pharmaceuticals on marine mammal leukocytes. In the environment, this exposure scenario is quite unlikely and would be occurring only in the case of accidental spills or illegal dumping of certain pharmaceuticals. Acute toxicity data give important baseline values for future analysis, and are practical to use for determining the mechanisms of action. But since pharmaceuticals are continuously discharged into the aquatic environment, a chronic rather than acute toxicity of certain APIs is to be expected and chronic toxicity studies are likely more environmentally representative (Crane *et al.*, 2006).

Chronic effects after exposure to pharmaceuticals have been established in freshwater organisms of lower trophic levels (Gagné *et al.*, 2006a, Gerhardt *et al.*, 2002, Isidori *et al.*, 2006). A variety of sublethal responses have been used as biomarkers to determine the impact of pharmaceutical exposure (Gagné *et al.*, 2006a, Laville *et al.*, 2004). In marine species, chronic toxicity of pharmaceuticals is, however, less studied (Aguirre-Martinez *et al.*, 2013a, Aguirre-Martinez *et al.*, 2013b, Aguirre-Martinez *et al.*, 2010, Buratti *et al.*, 2010, Martin-Diaz *et al.*, 2009b).

To investigate chronic effects of pharmaceuticals in marine mammals, particularly pinnipeds, it would be best to plan an extensive study on live and deceased animals. It would be preferable to

combine biomarkers like histopathology and immune markers with chemical analyses of different tissues to determine environmentally relevant levels of the xenobiotic cocktail that causes the observed effects. A meta-analysis could subsequently determine how e.g. contaminants, gender, and biomarkers are related to each other and to what extent pharmaceutical exposure can be correlated with decreased health parameters, if so.

More specifically, recently deceased animals could be utilized for organ-specific qualitative and quantitative chemical analyses of xenobiotics. To determine whether or not pharmaceuticals have the potential to accumulate in marine mammals, blubber and brain should be analyzed. Lipophilic pharmaceuticals have been shown to accumulate in liver and brain of fish more than any other tissue (Liu *et al.*, 2015). Excretory organs like liver and kidneys should be investigated, as well. Even if pharmaceuticals would not accumulate in marine mammals, the molecules will pass the excretory organs after being ingested.

Free-ranging animals could be utilized to obtain blood samples with cells that could be analyzed for their function *in vitro* as well as for chemical analysis of xenobiotics present in the blood. Impaired cell function and exposure concentration to the xenobiotic cocktail could therefore be correlated. Combining lymphocyte proliferation and cell surface markers would be an excellent model to determine possible impacts on host resistance (Luster *et al.*, 1992). If permits can be obtained, the gold standard of correlating exposure to pharmaceuticals and immunotoxic effects would be to capture seal pups and experimentally expose the animals in laboratory settings with fixed parameters.

This study could identify to what extent specific pharmaceuticals could produce adverse effects in comparison with legacy pollutants that should be qualified and quantified in parallel. This information is crucial to characterize and manage the associated environmental risk.

6.5 Importance of Marine Mammals as Sentinels in Toxicology

Marine mammals are important model organisms in toxicology due to their particular position in the ecosystem. Piscivorous aquatic mammals like harbor seals are particularly subject to xenobiotics because of their position in the food web, their habitat (e.g. estuaries) where contaminant concentrations are generally higher than in the open ocean, their longevity, adipose

deposits and their specific reproductive physiology such as mother-pup transfer of contaminants (Fossi *et al.*, 2003, Ross *et al.*, 1996b). Furthermore, harbor seals are non-migratory, allowing comparisons between sites with different contamination levels (Frouin *et al.*, 2010a).

The main focus of toxicological research has been the assessment of accumulation of legacy contaminants in marine mammals. Spatial and temporal studies have been conducted in the past decades and are still being published today. Even though evidence compiled that marine mammals are exposed to significant amounts of e.g. OCs, dose-response data are largely lacking particularly for immunotoxicity (Desforges *et al.*, 2016). Only around 50 studies have assessed the toxic effect of legacy contaminants on the marine mammals and apart from this work no studies have assessed the effect of pharmaceuticals on marine mammal immune cells.

Marine mammal models should be further developed in the future and cannot easily be replaced by more classic models like mice. Previous studies have shown that mice lymphocytes react very differently to OC exposure than lymphocytes of different marine mammal species (Mori *et al.*, 2006). Therefore, results obtained with mice would not necessarily predict toxicity towards marine mammals.

New methods are paving the way for research on untraditional model species. The study of non-model transcriptomes for instance is being transformed by massively parallel sequencing approaches like Roche 454 and Illumina HiSeq. These new methods allow an increased sequencing depth and coverage at relatively low cost (Vera *et al.*, 2008).

6.6 Cell Lines as Models for Marine Mammal PBMCs

Cell lines have been used to model parts of the physiology of organisms for several decades in toxicology. Although only displaying a small percentage of the metabolic activity of the whole organism they have been evaluated as useful, cost-effective tools in toxicology testing (Fent, 2001). The European Union and the REACH regulation (Registration, Evaluation, Authorization and Restriction of Chemicals) have even emphasized that alternative methods should be prioritized over animal toxicity tests whenever possible.

In this work, the harbor seal 11B7501 B lymphoma cell line that was utilized. It used to be commercially available at ATCC (as CRL-1940) and was developed by P. Marx and J. Kluge from blood of a male harbor seal with B lymphoma.

While it would have been possible to compare the sensitivity of the 11B7501 cell line and B lymphocytes within PBMCs to pharmaceuticals, we opted to test the proliferative responses of T lymphocytes in PBMCs instead for the following reasons. First of all, T lymphocytes have been found to be more sensitive to pollutant responses than B lymphocytes, and furthermore seem to be able to predict an effect on host resistance better (Luster *et al.*, 1993). In our study this was observed when comparing the lymphocyte proliferation inhibition of the B cell line with T lymphocytes with exposure to individual APIs. Secondly, the percentage of T lymphocytes in blood samples of seals in far superior to that of B lymphocytes. Therefore, we would have needed a higher volume of blood samples and a higher number of animals to conduct the same amount of analyses with B lymphocytes.

The ideal way to assess whether cell lines are representative of lymphocytes in free-ranging animals would be to compare both B and T cell lines with B and T lymphocytes. Commercially available marine mammal cell lines are, however, very rare and to our knowledge do not exist for T lymphocytes in harbor seals. It is possible to produce cell lines from primary cells, but this is a time and resource extensive process that was not possible during the course of this work. If marine mammal lymphocyte cell lines would be as sensitive as lymphocytes of free-ranging animals, they could present a cost effective tool for immunotoxicological studies in the future.

Section 7.

Contribution of this Work to the Advancement of Science

7 CONTRIBUTION OF THIS WORK TO THE ADVANCEMENT OF SCIENCE

In order to have effective risk assessment and management, it is critical to know the nature and magnitude of potential adverse health risks that an exposure to immunotoxic xenobiotics can have especially on highly exposed populations. The risk assessment of legacy contaminants in marine mammals is extensive, especially the determination of the accumulation and exposure concentrations of POPs in tissue. However, notably in the field of immunotoxicity dose-response data are limited. Emerging contaminants like pharmaceuticals are yet to be assessed in marine mammals for their accumlative potential and for environmentally relevant exposure concentrations. This work is the first to attempt the determination of dose-response curves and IC_{50} values for invididual and binary mixtures of pharmaceuticals.

To assess potential hazards, the establishment of threshold levels, LOECs and EC_{50} values are needed for relevant immune end points. Subsequently, effects at a molecular, cellular and individual level need to be extrapolated to the population level, which will then serve risk management and conservation plans (Desforges *et al.*, 2016).

It is yet too early to state that pharmaceuticals are immunotoxic to marine mammals in the environment. This study merely demonstrates that they do have the potential to inhibit lymphocyte proliferation, the cell cycle and induce genes like HSP70 at high exposure concentrations and short exposure times.

Moreover, even if pharmaceuticals would be proven to be harmful to the environment in the future, it is unlikely that the production or consumption would be regulated. Rather, education of the general public and an establishment of recycling programmes of expired, unwanted medication to avoid dumping in the sink, toilet or garbage could be a realistic solution to dealing with environmental contamination. Evolving processes in wastewater treatment plants could furthermore increase the removal of a wider variety of molecular structures that are excreted after application for human or veterinary processes.

Section 8.

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