INRS
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INSTITUT ARMAND-FRAPPIER

CONTAMINATION ET IMPACT IMMUNOLOGIQUE CHEZ TROIS ESPÈCES DE PHOQUES DU SAINT-LAURENT (*PHOCA VITULINA, HALICHOERUS GRYPUS* ET *PHOCA GROENLANDICA*)

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THÈSE PRÉSENTÉE POUR L'OBTENTION DU GRADE DE
PHILOSOPHIAE DOCTOR (PH.D.)
EN BIOLOGIE

JURY D'ÉVALUATION

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RÉSUMÉ GÉNÉRAL

L'utilisation croissante de substances polluantes par l'homme, leur présence persistante dans l'environnement marin malgré leur réglementation et leurs effets sur les organismes aquatiques constituent l'un des problèmes majeurs du XXIème siècle. Pour étudier l'impact des contaminants sur le système immunitaire des pinnipèdes, il était nécessaire dans un premier temps de mieux comprendre l'immunologie et l'exposition aux contaminants chez différentes espèces de phoques comme les phoques communs (*Phoca vitulina*), gris (*Halichoerus grypus*) et du Groenland (*Phoca groenlandica*).

Dans cette thèse nous avons déterminé que des changements en lien avec l'âge se produisaient dans le système immunitaire inné des pinnipèdes. Tandis que l'activité et l'efficacité phagocytaire augmentaient durant la lactation chez les phoques nouveau-nés, suggérant un système immunitaire compétent; chez les femelles adultes de phoque commun, l'activité phagocytaire diminuait avec l'âge. Ces résultats suggèrent que dépendamment de l'âge, la capacité des organismes à répondre aux pathogènes varie. De plus, des expériences réalisées *in vitro* chez un phoque gris ont permis de démontrer que les réponses diffèraient selon que les lymphocytes T provenaient du thymus, d'un ganglion lymphatique ou du sang, permettant d'envisager de déterminer une action spécifique d'un polluant donné sur la prolifération des lymphocytes T d'un tissu cible.

Dans un deuxième volet, un modèle biologique fut choisi, le phoque du Groenland, pour étudier le transfert des contaminants entre une mère et son jeune durant la lactation. Nos résultats démontrent que le transfert des pesticides, PBDE et BPC sont régis par leur liposolubilité, et qu'un transfert sélectif se produit durant la production du lait conduisant à des patrons de contaminants différents entre la mère et son jeune. De plus, l'analyse de PBDE dans le gras et le sérum des trois espèces de phoques de l'Estuaire et du Golfe du Saint-Laurent ont mis en évidence que les nouveau-nés provenant de l'Estuaire sont cinq fois plus contaminés que ceux du Golfe. Finalement, nous avons établi qu'il est envisageable d'utiliser les isotopes stables d'azote pour évaluer le transfert des butylétains à travers le réseau trophique de l'Estuaire du Saint-Laurent.

Dans un troisième temps, nous avons mis en évidence lors de travaux *in vitro* réalisés chez le phoque commun que les fonctions immunitaires testées (phagocytose, prolifération lymphoblastique et activité cytotoxique des cellules NK) étaient plus touchées par l'exposition aux butylétains chez les adultes que chez les nouveau-nés. Cependant, en présence de congénères de PBDE la flambée oxydative, le niveau intracellulaire de thiols et l'activité et l'efficacité phagocytaire présentaient des réponses similaires entre les adultes et les nouveau-nés et une lignée cellulaire issu d'un lymphome B de phoque commun. Avec cette dernière, nous avons pu démontrer qu'à des niveaux environnementaux (retrouvés dans le sang des pinnipèdes), le B[a]P, le sélénium, le vanadium et l'arsenic induisaient aussi bien des effets immunotoxiques (diminution de la prolifération lymphoblastique et de la phagocytose) que génotoxiques (induction de micronuclei).

Dans l'ensemble, nos travaux suggèrent que le système immunitaire du phoque commun peut-être affaibli suite à l'exposition permanente aux contaminants retrouvés dans son environnement. De plus, il ressort que les nouveau-nés sont exposés aux contaminants durant un stade critique de leur développement.

Étudiant **Directeur de recherche**

REMERCIEMENTS

Je tiens à exprimer toute ma gratitude aux personnes qui ont accepté d'être examinateurs pour ce travail.

A la Pre Cathy Vaillancourt. Merci pour le covoiturage, les échanges d'idées et les longues discussions sur nos filles « Pitoune » et « Lilou » (nos chats!). Merci Cathy, pour avoir accepter de t'impliquer dans ce travail en devenant la présidente de jury de thèse malgré tes nombreuses obligations.

Au Pr.Sylvain De Guise. Notre première rencontre remonte à novembre 2007. J'étais alors impressionnée de rencontrer l'auteur des nombreuses publications que j'avais lues avec un vif intérêt. Étant un ancien étudiant du Professeur Michel Fournier, avec sa participation à ce jury, la boucle est bouclée, en quelque sorte.

Je suis très heureuse de pouvoir compter parmi les membres de mon jury la Pre Tatiana Scorza. Tatiana avait déja témoigné de son intérêt pour ce travail en acceptant préalablement d'être membre du jury lors de mon séminaire de recherche.

Mes plus vifs remerciements et toute ma reconnaissance vont à mes responsables scientifiques.

Tout d'abord, au Pr. Michel Fournier, mon directeur de thèse, sans qui cette aventure n'aurait jamais vu le jour. Après m'avoir connu durant ma maîtrise à Rimouski, il n'a pas hésité à me proposer un sujet libre sur les pinnipèdes. Merci pour la confiance et le soutien dont tu as fait preuve à mon égard.Tu m'as également permis de m'affirmer en tant que chercheuse, en me faisant rencontrer de nombreuses personnes par les diffèrents congrès où tu as accepté de m'envoyer et en me permettant de faire mes propres choix quand à l'orientation de mon sujet. Je tiens également à souligner sa disponibilité et sa patience dans les situations « de dernières minutes » auxquelles je l'ai de temps à autres confronté et qui m'auront valu le surnom « de terreur de Vendée»...

Merci également au Dr. Michel Lebeuf, mon codirecteur de thèse, qui avait par le passé accepté d'être mon maître de stage quand, déjà, je voulais travailler sur les mammifères marins. Son esprit critique et sa rigueur ont été de précieux enseignements. Merci de m'avoir accueilli dans ton laboratoire à l'Institut Maurice-Lamontagne et de m'avoir initié au monde fascinant de la chimie. Merci pour les bons moments passés à Terre-Neuve lors de notre échantillonnage de phoques communs et à Cape Town lors du congrès de la SMM en 2007.

Bien sûr ce projet « phoque » n'aurait jamais pu être initié sans le soutien d'un spécialiste des pinnipèdes, le Dr. Mike Hammill du MPO (IML). Il m'a donné l'occasion de participer à de nombreux terrains avec lui et m'a enseigné les techniques de captures, de biopsies, et de collecte de sang et de lait sur les phoques communs, gris et du Groenland. Merci pour tous les bons moments passés sur le terrain (notamment la crème glacée au phoque!!) et au retour de terrain.

Au Dr. Émilien Pelletier de l'ISMER, je tiens à exprimer toute ma reconnaissance pour avoir accepter de collaborer à la partie butylétain de ce projet et de m'avoir permis l'accès à son laboratoire lorsque je suis venu faire du terrain au Bic et que je me suis retrouvée « SLF » (sans laboratoire fixe).

Mes expériences en laboratoire ont été grandement facilitées par les personnes suivantes. Marlène Fortier de l'INRS-IAF, une assistante de recherche en or qui m'a fait profiter de son expérience en cytométrie de flux. Steve Trottier de l'IML, le grand manitou de la chimie qui a toujours eu la patience de répondre à mes questions sur le fonctionnement des appareils et l'analyse des résultats. Le Dr. Richard St.-Louis de l'ISMER qui m'a initié à l'analyse des butylétains, et qui a su prendre sur son temps précieux pour m'expliquer les rouages de la chimie. Enfin à Tiphaine Brett, qui au cours de son stage, a pu m'aider à venir à bout de tous mes échantillons pour la partie « chimie » de mon projet.

Les différents échantillonnages sur le terrain n'auraient pu être réalisés sans la collaboration de nombreuses personnes. Tout d'abord, la Dre. Becky Sjare qui a initié le projet phoque commun à Terre-Neuve avec le Dr. Michel Lebeuf et sur lequel je suis venu me greffer par la suite. L'équipe mammifères marins de l'IML, avec Jean-Francois Gosselin qui nous à aider lors de la capture des phoques communs au Bic, Samuel Turgeon qui fut d'une aide plus que précieuse pour les terrains phoques gris et du Groenland (Île du Prince Édouard et Îles de la Madeleine) et Emmanuel Chasson pour son aide durant la capture des phoques gris (Île du Prince Édouard). Enfin merci à Harrison MacRae, notre pilote d'hélicoptère et aide terrain pour les captures de phoques gris et Wayne Penney pour la cueillette des phoques communs à Terre-Neuve.

Je tiens aussi à remercier, le Dr. Édouard Potworowski pour ses critiques constructives au niveau de la rédaction des manuscripts.

Merci à tous les gens passés ou présents au laboratoire, pour l'ambiance, les échanges et les encouragements. Un merci particulier à Émilie, Audrey, Sophie et Marie pour vôtre amitié.

J'adresse de sincères remerciements à l'équipe administrative avec notamment: Jocelyne Ash pour ta patience et ta disponibilité, pour ta bonne humeur. Anne Philippon pour le suivi de mon dossier étudiant et ta patience face à toutes mes questions concernant la partie administrative du doctorat. A l'équipe de la bibliothèque pour mes nombreuses listes de demande d'articles et la rapidité avec laquelle vous les obteniez. Merci également à Josée Labonne pour m'avoir aidée dans le casse-tête que pouvait représenter les commandes de certains produits.

Merci également à la Fondation Armand-Frappier pour le soutien financier.

Quand ma rédaction est devenue trop rébarbative, merci à Richard Sears de m'avoir accueilli au sein du MICS. Merci à toute l'équipe pour m'avoir sorti la tête de l'ordinateur et m'avoir initié aux joies du terrain avec les cétacés, les approches en zodiac et par la suite, le traitement des photos et la photo-identification. Un merci particulier à Christian Ramp (« Doudou »), Fred Paquet, Charlène Jourdes (« Morfalette »), Petra Reimann, Kathy Gavrilchuk (« The Ed »), Cécile Dars (« Dee »), Boris (« Momo ») et David (« Grand Morfale »).

Ma vie montréalaise n'aurait pas été aussi agréable sans les deux merveilleux colocs que j'ai eu la chance de cotoyer pendant deux ans et demi. Merci à François Desilets et Sébastien Robert pour m'avoir accueilli et m'avoir initié aux joies du Québec. Avec notamment les matchs de Hockey qui se terminaient généralement par une tournée dans les bars avec les amis: Tommy et Dominica, Caroline et Pat et Sébastien et Julia.

Un gros merci aux amis du Québec pour avoir été là dans les bons moments comme dans les moments de doute, merci à Nathalie (« Nath ») et Frank (qui malheureusement s'en sont retournés en France) à Charles et Annabelle (pour les partys mémorables), à Julien (« Juju »), à Aurélie (« la noune ») pour la grande visite, à Catherine (« cathou ») et Mathieu et leur adorable Marie, à Guillaume pour m'avoir toujours héberger quand je venais à Rimouski, à Mathieu (« Matmat ») pour nos soirées cinéma, à Stephanie (« Steph' ») pour un petit café sur Montréal, à Pierre Saint-Laurent (« Pierrot ») pour nos retrouvailles sur Rimouski ou Halifax et à Virginie (« Vivi ») pour son soutien durant la thèse, continues à t'accrocher un jour on en voit le bout de cette thèse (la preuve!).

Merci également aux amis de France qui tiennent malgré la distance, une pensée pour Marianne et Christophe (« Mimi » et « Cricri »), Émilie, Ludivine et Jeremy (« Lulu » et « Jerem' »), Dorothée, Michael et Caroline (« Mitch » et « Caro ») et les nouveaux

amis d'Halifax : Del et Frances, Mario, Julien et Nina (et la petite Nolwen) et Khalida, une amie fraîchement arrivée de Rimouski.

Enfin merci à toute ma famille, sans qui je ne serais bien sûr jamais arrivée là. Merci pour votre aide et votre soutien, pour avoir toujours cru en moi et pour avoir toujours essayé de comprendre sur quoi je travaille. Ça y est je vais arrêter de vous embêter avec ma thèse, ce sera maintenant la recherche d'un post-doctorat qu'il faudra supporter.

Ma dernière pensée va bien sûr à celui qui occupe mon coeur, Xavier. Merci pour ta présence, ton soutien au quotidien et ta patience face à mon stress de thésarde.

(Première étape trouver un post-doctorat!!)

« Des chercheurs, on en trouve, mais des trouveurs, on en cherche. »

Général de Gaulle

« Vous savez ce que c'est que la recherche : on part sur une question et on trouve en cours de route des faits qui vous en posent une autre »

Philippe Meyer

« If everything seems to be going well, you have obviously overlooked someting »

Murphy's law

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LISTE DES ABBRÉVIATIONS

B[a]P : benzo[a]pyrene
BPC : Biphényles Polychlorés
Cellule NK : cellule *Natural Killer*
DBT : Dibutylétain
EMSL : Écosystème Marin du St-Laurent
EOR : Espèces oxygénées réactives
ESL : Estuaire du St-Laurent
Hg : *Halichoerus grypus* (Phoque gris)
ml : millilitre
mM : millimolaire
MBT : Monobutylétain
PCB : Polychlorinated biphenyls
PBDE : polybromés diphényles éthers
PCDE: polychlorodiphényles éthers
Pg : *Phoca groenlandica* (Phoque du Groenland)
Pv : *Phoca vitulina* (Phoque commun)
SLE : St. Lawrence Estuary
TBT : Tributylétain
µg : microgramme
µL : microlitre
µM : micromolaire

INTRODUCTION

Les océans ont longtemps été considérés comme des puits sans fond dans lesquels les déchets domestiques et industriels pouvaient être déversés. Il n'est donc pas surprenant qu'aujourd'hui on mesure des concentrations significatives de divers contaminants dans les différents compartiments de l'environnement marin (eau, sédiments et organismes). Ceci est particulièrement vrai pour les composés stables, tels que les organochlorés et les organobromés, lesquels persistent longtemps après leur production et ont seulement été réglementés récemment (Ross et al., 2009).

Les mammifères marins, étant au sommet de la chaîne alimentaire, accumulent de grandes quantités des contaminants présents dans leur environnement. Les organochlorés (BPC et pesticides chlorés), les organobromés (PBDE), les métaux lourds et les hydrocarbures aromatiques polycycliques (HAP) se retrouvent chez ces animaux et sont d'un intérêt particulier à cause de leur abondance et/ou de leur toxicité connue. Leurs effets, leurs distributions et leurs transmissions restent largement méconnus chez les mammifères aquatiques. Cependant, certains de ces contaminants connus pour être immunosuppresseurs chez les animaux de laboratoire et les humains, ont été pointés du doigt et suspectés d'avoir contribué aux mortalités élevées lors des nombreuses épizooties qu'ont subis les mammifères marins, ces vingt dernières années. De récentes études ont effectuées des comparaisons entre les mammifères marins et le modèle murin, il en ressort que les effets des organochlorés sur la prolifération des lymphocytes T et B (Mori et al., 2006; 2008) et sur la modulation de la flambée oxydative (Levin et al., 2007) diffèrents entre les deux modèles.

Trois thèmes majeurs ont été choisis pour répondre à la problématique « contaminants et mammifères marins ». Cette thèse sera ainsi construite par article selon les axes « Immunologie comparée », « Dynamique des contaminants » et « Toxicologie ». Pour chaque axe (chapitre) les sous-parties ont été rédigées sous forme d'articles pour faciliter la publication dans des journaux scientifiques à comité de lecture. Il en résulte que certaines répétitions peuvent se produire au sein d'un même chapitre, spécialement en ce qui à trait aux revues de littérature et à la partie matériel et méthodes. Dans la revue de littérature, le résumé sur la toxicologie des organoétains chez les organismes marins est developpé plus en détail dans la partie 2. Ce chapitre intitulé « Toxicology of organotins in marine organisms : a review » fait l'objet d'une publication dans le livre intitulé « Organometallic compounds : Preparation, Structure and Properties ».

Dans un premier temps, ce travail vise à mieux définir les différences dans les réponses immunitaires entre plusieurs espèces de phoques et plusieurs organes du système immunitaire. Deux études ont été réalisées pour répondre à ces objectifs et sont présentés dans le chapitre « Immunotoxicologie comparée ».

Objectif 1 : Évaluer l'évolution d'un paramètre du système immunitaire inné, la phagocytose, avec l'âge chez les femelles phoque commun et évaluer son évolution durant la lactation chez trois espèces de phoques (commun, gris et du Groenland).

Objectif 2 : Déterminer si les réponses mitogéniques des lymphocytes T, provenant de différents organes immunitaires (sang, thymus, ganglions lymphatiques), exposés à divers contaminants (métaux lourds et BPC) sont similaires.

Deux études ont été réalisées pour répondre à ces objectifs et sont présentées dans le chapitre « Immunologie comparée ».

L'article intitulé « Phagocytosis in pup and adult harbour, grey and harp seals » a été accepté dans la revue à comité de lecture « Veterinary Immunology and Immunopathology » (Chapitre 3.1).

L'article intitulé « T lymphocyte-proliferative responses of a grey seal (*Halichoerus grypus*) exposed to heavy metals and PCBs *in vitro* » sera soumis sous peu à la revue à comité de lecture « Marine Pollution Bulletin » (Chapitre 3.2).

Ces deux études visent à mieux comprendre la réponse de deux éléments majeurs du système immunitaire (la phagocytose et la prolifération lymphoblastique) chez les phoques. Cette étape est préalable à l'étude du potentiel immunotoxique de l'exposition à diverses familles de contaminants.

Dans un deuxième temps, cette thèse vise à mieux évaluer la dynamique des contaminants entre la mère et son jeune mais également à travers le réseau trophique. Les objectifs sont :

Objectif 3 : Définir le transfert des PBDE, BPC et pesticides chlorés durant la lactation chez le phoque du Groenland

Objectif 4 : Caractériser les concentrations de PBDE chez les nouveau-nés de trois espèces de phoques (gris, commun et du Groenland) dans l'Estuaire et le Golfe du St.-Laurent.

Objectif 5 : Estimer le transfert des butylétains à travers le réseau trophique du St.-Laurent.

Trois études ont été réalisées pour répondre à ces objectifs et sont présentées dans le chapitre « Dynamique des contaminants ».

L'article intitulé « Dynamics of contaminant transfer from mother to pup during lactation in harp seals *Phoca groenlandica* » sera soumis sous peu à la revue à comité de lecture « Chemosphere ». (Chapitre 4.1).

L'article intitulé « PBDE in serum and blubber of pup harbour, grey and harp seals from Eastern Canada» sera soumis sous peu à la revue à comité de lecture « Environmental Science and Technology ». (Chapitre 4.2).

L'article intitulé provisoirement « Butyltins in a Food Web from St. Lawrence Estuary, Quebec, Canada » sera un plus développé par la suite (pour l'instant il ne s'agit que d'une étude préliminaire) et sera soumis pour publication. (Chapitre 4.3).

Mieux comprendre la dynamique régissant le transfert et la distribution des contaminants à travers le réseau trophique, pourrait permettre de mieux prévoir les niveaux d'exposition chez les animaux. De même, évaluer la dynamique du transfert durant la lactation, donne l'information sur l'évolution de la charge en contaminants chez la mère et le jeune. Enfin, mesurer les concentrations de contaminants chez les nouveau-nés dans différentes localisations permet de mieux définir, comprendre et interpréter les facteurs influençant les niveaux de contaminants chez les différentes espèces de phoques.

Dans un troisième temps, cette thèse vise à mieux comprendre le potentiel immunotoxique de diverses familles de composants PBDE, métaux lourds et HAP.

Objectif 6 : Déterminer le potentiel immunotoxique des butylétains (TBT, DBT et MBT) chez les adultes et nouveau-nés de phoque commun.

Objectif 7 : Déterminer le potentiel immunotoxique des PBDE chez les adultes et nouveau-nés de phoque commun et d'une lignée cellulaire de lymphoblastes issue d'un phoque commun.

Objectif 8 : Évaluer le potentiel immunotoxique des métaux lourds (fer, argent, zinc, chrome, vanadium, sélénium et arsenic) et d'un HAP (le benzo[a]pyrène) sur la lignée cellulaire de lymphoblastes issue d'un phoque commun.

Trois études ont été réalisées pour répondre à ces objectifs et sont présentées dans le chapitre « Toxicologie ».

Les résultats de l'objectif 7 ont été publiés comme suit : Frouin H., Lebeuf M., St.-Louis R., Hammill M.,Pelletier É., Fournier M., 2008. Toxic effects of tributyltin and its metabolites on harbour seal (Phoca vitulina immune cells *in vitro*. Aquatic Toxicology 90, 243-251 (Chapitre 5.1).

Les résultats de l'objectif 4 ont fait l'objet d'un article intitulé « Effects of individual polybrominated diphenyl ether (PBDE) congeners on harbour seal immune cells *in vitro* » et accepté dans la revue à comité de lecture « Marine Pollution Bulletin » (Chapitre 5.2).

Les résultats de l'objectif 5 ont fait l'objet d'un article intitulé « Toxic effects of various pollutants in 11B7501 lymphoma B cell line from harbour seal (*Phoca vitulina*) » et soumis à la revue à comité de lecture « Toxicology » (Chapitre 5.3).

Ces trois études visent à enrichir notre connaissance actuelle du potentiel immunotoxique de différents contaminants chez le phoque commun afin de mieux comprendre le lien entre la santé et le niveau de contamination. En effet, lors des épizooties à morbillivirus en 1988 et 2002, en Europe, les phoques les plus sévèrement touchés étaient ceux dont les niveaux de contaminants étaient les plus élevés. Une relation entre l'exposition à des contaminants et une diminution de la performance immunitaire a donc été suggérée pour expliquer ce phénomène. En dépit d'un intérêt croissant pour l'immunotoxicologie chez les mammifères marins, certaines familles de contaminants ont été peu ou pas étudiées. Les butylétains sont des contaminants qui persistent dans l'environnement marin, malgré leur réglementation dans les années 70, et leurs effets sont bien connus chez les invertébrés marins; cependant ils ont fait l'objet de peu d'études dans les niveaux supérieurs de la chaîne trophique. Les PBDE sont des contaminants émergents de ces deux dernières décennies et bien que de nombreuses études aient été réalisées sur les mammifères terrestres (souris, rats et humains en particuliers) conduisant récemment à leur réglementation, l'évaluation de leurs effets immunotoxiques chez les mammifères marins fait encore défaut. Enfin, l'effet de certains métaux chez les mammifères aquatiques ont été intensément étudiés (notamment le mercure) tandis que d'autres ont été plus ou moins ignorés.

Tous les travaux présentés ici sont issus de mes propres recherches, à l'exception de l'article intitulé « T cell-proliferative responses of grey seal (*Halichoerus grypus*) exposed to contaminants *in vitro* » qui résulte de travaux réalisés en 2000 par l'équipe du laboratoire du Professeur Michel Fournier et dont j'ai traité et interprété les résultats. La contribution des auteurs pour les différents articles est la suivante : le Pr. Michel Fournier (INRS-Institut Armand-Frappier) et le Dr. Michel Lebeuf (Institut Maurice-Lamontagne) ont supervisé cette thèse, fourni un support logistique pour les analyses biologiques (M.F. à l'Institut National de la Recherche Scientifique-Institut Armand-Frappier) et chimiques (M.L. à l'Institut Maurice-Lamontagne), ainsi que dispensé leurs

conseils et leurs expertises tout au long du doctorat. Le Dr. Mike Hammill (Institut Maurice-Lamontagne) a fourni un support logistique pour les échantillonnages de phoques et a partagé son expertise en mammalogie marine. Enfin le Pr. Émilien Pelletier (UQAR-ISMER) et l'associé de recherche, le Dr. Richard Saint-Louis (UQAR-ISMER), ont fourni un support logistique pour l'analyse chimique des butylétains et ont partagé leur expertise en chimie.

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1. REVUE DE LITTÉRATURE

1.1. Les phoques du Saint-Laurent

Quatre représentants de la famille des phocidés (ordre des Pinnipèdes) sont communément retrouvés dans l'écosystème marin du St.-Laurent (EMSL). Il s'agit du phoque commun (*Phoca vitulina*), du phoque gris (*Halichoerus grypus*), du phoque du Groenland (*Phoca groenlandica*) et du phoque à capuchon (*Cistophora cristata*). Puisque dans notre étude, seules les trois premières espèces étaient étudiées, le phoque à capuchon ne sera pas discuté dans cette section.

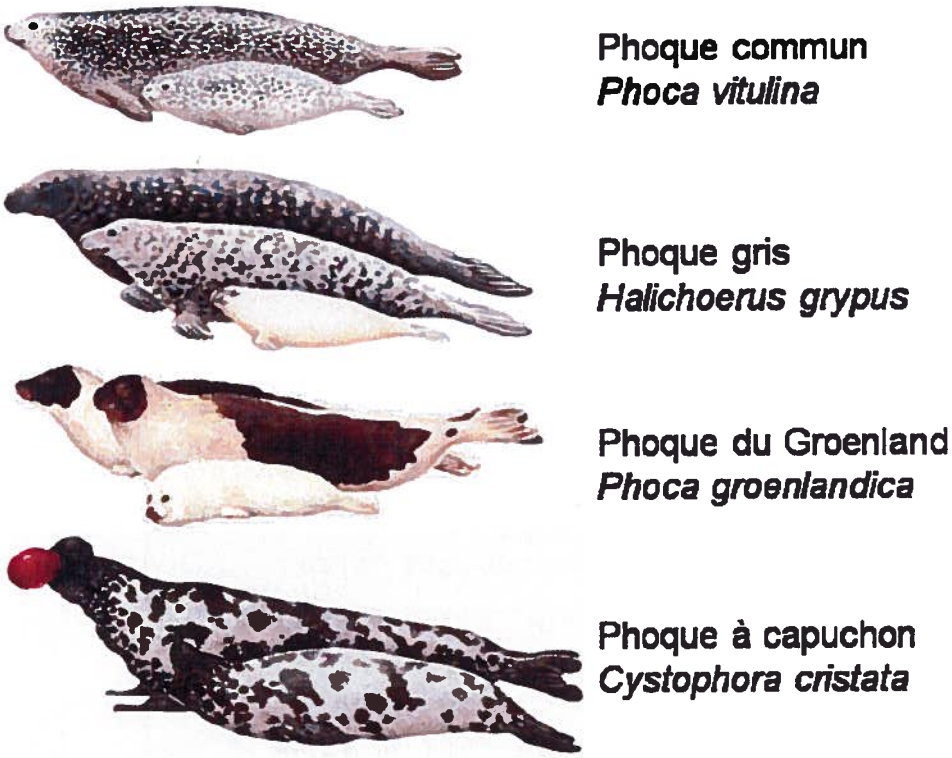

Figure 1. Illustration des quatre espèces de phoques retrouvées dans l'Estuaire et le Golfe du Saint-Laurent (images issues du web).

1.1.1. Distribution

Le phoque commun est un petit phoque (<120 kg) comprenant cinq sous-espèces, lesquelles sont abondamment distribuées le long des côtes de l'hémisphère nord (Burns, 2002). Dans l'Atlantique canadien, les phoques communs (*P. vitulina concolor*) sont des résidents permanents, dispersés en plusieurs colonies dans l'Estuaire et le Golfe du St.-Laurent (Lebeuf et al., 2003; Lesage et al., 2004). Bien que

supposément sédentaires tout au long de l'année (Lowry et al., 2001), les adultes réalisent en fait des migrations sur de longues distances dans le cadre de l'activité/distribution saisonnière (Lesage et al., 2004).

Le phoque gris (poids compris entre 170 et 300 kg) se retrouve également dans les zones côtières de l'Atlantique Nord. En hiver, les phoques gris migrent dans le Sud du Golfe et à l'île des Sables pour la période de mise bas/reproduction (Mansfield and Beck, 1977). A l'été, pendant ou peu après leur période de mue (entre mai et juin) les phoques vont quitter leur site de reproduction et se disperser le long de la côte nord-ouest des États-Unis, les côtes de la Nouvelle-Écosse et de Terre-Neuve, à travers le Golfe du St-Laurent et pénétrer dans l'Estuaire du St-Laurent (Stobo et al., 1990; Lavigeur and Hammill, 1993).

Le phoque du Groenland (~ 135 kg) a une population estimé à 5,2 millions dans l'Atlantique du nord-ouest. Il est considéré comme un résident saisonnier de l'EMSL. Cette espèce passe l'été dans l'Arctique canadien et le long de la côte du Groenland et migre à la fin de l'automne vers le sud pour passer l'hiver dans le Golfe du St-Laurent et à l'extérieur de la côte nord-est de Terre-Neuve (Sergeant, 1965).

1.1.2. Alimentation

Proie (espèce)	Part que représente chaque proie dans le régime alimentaire du phoque commun	gris	du Groenland
Hareng atlantique (*Clupea harengus*)	67,8%	11,9%	2,6%
Morue de l'Arctique (*Boreogadus saida*)			9,4%
Flétan du Groenland (*Reinhardtius hippoglossoides*)		0,1%	3,9%
Morue de l'Atlantique (*Gadus morhua*)	15,9%	24,1%	4,9%
Lançon (*Ammodytes* spp.)		58,4%	18,7%
Capelan (*Mallotus villosus*)	15,3%	4,8%	54,5%

Tableau 1. Consommation estimée des principales proies dans le régime alimentaire de chacune des trois espèces de phoques (données obtenues en 1996 dans le Golfe du St.-Laurent) (calculé à partir de Hammill et Stenson, 2000).

La consommation des proies a été analysée par Hammill et Stenson (2000) pour les quatre espèces de phoques occupant l'Atlantique canadien. En analysant la consommation totale des proies, 77% (estimé sur la base du poids) s'avère être

composé de poissons. Le capelan et le lançon en sont les espèces dominantes représentant 49% de la consommation totale de poissons. Les phoques du Groenland sont les consommateurs les plus importants, représentant 82% de la consommation totale, suivi par les phoques à capuchons (10% de la consommation totale), les phoques gris (7,8%) et les phoques communs (0,2%). La part occupée dans le régime alimentaire du phoque par chacune des principales espèces de poissons consommées est présentée dans le tableau 1.

1.1.3. Reproduction

Chez le phoque commun, l'accouplement s'effectue normalement juste avant le sevrage des chiots ou immédiatement après (Fisher, 1954) et il a souvent été suggéré qu'il était l'événement déclencheur qui pousse la femelle à quitter son chiot. Dans l'estuaire du St-Laurent, l'accouplement semble commencer au début juin et peut se prolonger jusqu'au début août, mais il est difficile d'évaluer précisément la date du début et de la fin de l'accouplement puisqu'il s'effectue normalement dans l'eau (Stirling, 1983). Suite à la fécondation, l'embryon ne s'implante pas immédiatement dans l'utérus, mais rentre en diapause durant environ 2 à 3 mois, ce qu'on appelle l'implantation différée (Fisher, 1954; Boulva and McLaren, 1980). Après cette diapause la croissance de l'embryon débute et se prolongera pendant environ 8 à 9 mois. Ainsi, la période de gestation, de la fécondation à la naissance, s'étend sur une période d'environ 11 mois. Dans l'estuaire du St-Laurent, la période des naissances débute vers la mi-mai et se poursuit jusqu'à la mi-juin (Dubé et al., 2003). À la naissance, le chiot pèse en moyenne 11 kg et le taux de croissance journalier moyen est estimé à 0,5 kg/jour (Dubé et al., 2003). Dans l'estuaire du St-Laurent, la période de lactation est estimée durer 34 jours (Dubé et al., 2003), ce qui la rend plus longue que la moyenne de 24 jours à l'Île des Sables (Muelbert and Bowen, 1993; Bowen et al., 2001) mais similaire aux 31-32 jours calculés pour les phoques communs en Colombie-Britannique (Cottrell and Ross, 2002).

Chez le phoque gris, la période de mise bas se déroule de la fin décembre au début février sur la banquise ou sur la terre ferme. Deux concentrations majeures de jeunes sont reconnues, sur l'Île des Sables à 250 km des la côte est de la Nouvelle Écosse et sur la banquise dans le sud du Golfe du St-Laurent (Mansfield et Beck, 1977). La période de reproduction débute avec la naissance du jeune. Le cycle de reproduction

comporte également une diapause embryonnaire d'environ 3 mois (Atkinson, 1997). Après cette diapause embryonnaire, la gestation se poursuit pendant environ 8 mois. Ce cycle permet ainsi de synchroniser les naissances avec l'accouplement. À la naissance, le chiot pèse en moyenne 16 kg et le taux de croissance journalier moyen est estimé à 2,8 kg/jour (Bowen et al., 1992b). La lactation dure entre 16 et 18 jours (Boness and James, 1979).

Chez le phoque du Groenland, la période de mise bas prend place dans le Golfe du St-Laurent début mars et s'effectue sur la banquise (Lydersen and Kovacs, 1999). La période de reproduction débute avec la naissance du jeune. Le cycle de reproduction comporte également une diapause embryonnaire d'environ 2-3 mois (Atkinson, 1997). Après cette diapause embryonnaire, la gestation se poursuit pendant environ 8 mois. A la naissance, le chiot pèse en moyenne 10,8 kg et naît avec un pelage blanc (lanugo) et pas de lard (Stewart and Lavigne, 1980). Durant les 12 jours de lactation, le taux de croissance journalier moyen du nouveau-né est estimé à 2,5 kg (Stewart and Lavigne, 1980; Kovacs and Lavigne, 1985).

1.2. Système immunitaire des mammifères marins

La connaissance du système immunitaire chez les mammifères marins a été améliorée en grande partie par les recherches en immunotoxicologie menées suite aux sévères épizooties encourues chez ces animaux. L'immunologie des mammifères marins a été préalablement résumée dans De Guise et al. (2003), De Guise (2005), Ross et al. (2003) et Ross et De Guise (2007). Cependant, l'immunologie du développement et la comparaison entre les organes immunitaires sont méconnus et nécessitent de nouvelles études.

1.2.1. Immunologie des mammifères marins

Le système immunitaire des mammifères est constitué de deux branches : le système immunitaire inné ou naturel et le système acquis ou adaptatif. L'immunité innée, bien que peu spécifique, constitue la première ligne de défense contre les infections. L'immunité adaptative répond avec un haut degré de spécificité et possède la propriété de « mémoire ». La mémoire immunologique représente la capacité du système immunitaire acquis de répondre plus rapidement et plus intensivement à un antigène

rencontré pour la seconde fois, tandis que le système immunitaire inné répond toujours à un antigène comme s'il s'agissait de la première fois. Les systèmes immunitaires inné et acquis ont été divisés en deux branches, l'immunité à médiation cellulaire et l'immunité humorale.

Les immunoglobulines, ou anticorps, sont des protéines solubles utilisées par le système immunitaire pour reconnaître et participer à l'élimination des antigènes. Les immunoglobulines ont été caractérisées aussi bien chez les cétacés que les pinnipèdes (Boyden and Gemeroy, 1950; Travis and Sanders, 1972; Nash and Mack, 1977; Cavagnolo, 1979; Andresdottir et al., 1987). Des anticorps mono- et polyclonaux ont été produits pour quantifier les immunoglobulines chez les phoques gris et communs, l'éléphant de mer boréal (*Mirounga angustirostris*) l'orque (*Orcinus orca*), le grand dauphin (*Tursiops truncatus*) et les loutres de mer du Nord (*Enhydra lutris lutris*) et du Sud (*Enhydra lutris nereis*) (Carter et al., 1990; King et al., 1993; 1994; 1998; Taylor et al., 2002; Beck and Rice, 2003).

Les lymphocytes sont les acteurs majeurs du système immunitaire acquis. Ils sont constitués de deux populations indifférentiables morphologiquement, les lymphocytes T et B, qui interviennent, respectivement, dans l'immunité à médiation cellulaire et l'immunité humorale. Des anticorps monoclonaux à réactivité croisée ont été utilisés afin d'identifier les sous-classes de lymphocytes chez les mammifères marins, par cytométrie en flux. Chez le grand dauphin, la réaction croisée a été démontrée en utilisant les anticorps dirigés contre le complexe majeur d'histocompatibilité (CMH) humain de classe II (Romano et al., 1992). De même, chez le béluga (*Delphinapterus leucas*), des tests de réaction croisée ont été effectués avec de nombreux anticorps (De Guise et al., 1997a). De plus, des anticorps mono- et polyclonaux (spécifiques à une espèce) dirigés vers les antigènes à la surface des cellules de mammifères marins ont été produits. Ainsi spécifiquement pour les cellules de cétacés, des anticorps dirigés contre des homologues de la molécule CD2 (exprimée par les lymphocytes T), des molécules CD19 et CD21 (exprimées par les lymphocytes B), de la molécule CD45 (un marqueur de l'activation des cellules T), et de l'intégrine β2 (un marque de l'activation des neutrophiles, monocytes et cellules B) ont été caractérisés par De Guise et al. (1998; 2002; 2004). Un anticorps monoclonal a également été développé pour caractériser spécifiquement les neutrophiles chez le grand dauphin (Kato et al., 2009).

Récemment, des travaux ont été réalisés sur l'analyse génétique des cytokines chez les cétacés et le phoque gris (Inoue et al., 1999a; Inoue et al., 1999b; Inoue et al.,

1999c; St-Laurent et al., 1999; St-Laurent and Archambault, 2000; Inoue et al., 2001; Shoji et al., 2001; Funke et al., 2003; Itou et al., 2003). Sitt et al. (2008) ont comparé les réponses de leucocytes mononucléaires induites par un mitogène entre le grand dauphin, le dauphin à flancs blancs du Pacifique (*Lagenorhynchus obliquidens*) et le béluga. Ils ont démontré que les réponses étaient similaires entre les trois cétacés avec une régulation soit à la hausse des gènes des cytokines IL-2, IL-4, IL-13 et IFNγ ou à la baisse des gènes de cytokines IL-12, IL-18, TGFβ.

La prolifération lymphocytaire représente une des actions faisant suite à l'activation des lymphocytes. Le test de transformation lymphoblastique (TTL) ou blastogenèse, induite *in vitro* par un mitogène, mesure, par l'incorporation d'agents marqués (comme la thymidine tritiée) dans le noyau de cellules en division, la capacité des lymphocytes à proliférer en réponse à une stimulation polyclonale non-spécifique. Ce test a été effectué chez de nombreuses espèces de cétacés et pinnipèdes (Mumford et al., 1975; Colgrove, 1978; De Swart et al., 1993; Ross et al., 1993; DiMolfetto-Landon et al., 1995; Lahvis et al., 1995; De Guise et al., 1996).

Les neutrophiles, qui représentent la première ligne de défense du système immunitaire inné, vont ingérer les particules étrangères par la phagocytose, et vont détruire les particules phagocytées grâce à des processus biochimiques (flambée oxydative). La phagocytose et la flambée oxydative ont été déterminées chez une grande variété d'espèces de mammifères marins (De Guise et al., 1995; Pillet et al., 2000; Itou et al., 2001; Noda et al., 2003; Levin et al., 2007).

Enfin, les lymphocytes NK (Natural Killer) sont capables de reconnaître les cellules cancéreuses et les cellules infectées par des virus et de les détruire. Des tests portant sur l'activité cytotoxique des cellules NK ont été développés chez le béluga (De Guise et al., 1997b) et les phoques commun (De Swart et al., 1994; Ross et al., 1996b) et gris (Hammond et al., 2005).

1.2.2. Immunologie du développement chez les mammifères marins

Peu de recherches ont été menées sur la maturation des fonctions immunitaires chez les mammifères marins en développement. Cependant Ross et al. (1993; 1994) ont établi que les niveaux d'immunoglobulines G (IgG) chez le nouveau-né phoque commun augmentaient durant la lactation, passant de 5% à la naissance (exprimé sur les niveaux maternels d'IgG) à 65% à 15 jours. Chez le phoque gris, un patron similaire était

observé, de faibles niveaux d'IgG et d'IgM étaient mesurés chez les nouveau-nés puis les niveaux augmentaient durant la période de lactation (Carter et al., 1990; King et al., 1994). Hall et al. (2003a) démontrèrent également une augmentation des IgG durant la lactation aussi bien chez le phoque gris, que l'éléphant de mer austral (*Mirounga leonina*). L'étude de Ross et al. (1994) suggérait que la prise du colostrum représente une source importante de transfert d'anticorps chez les nouveau-nés phoque commun. Une augmentation plus lente des niveaux d'IgA, IgG et IgM était observée chez les otaries à fourrure (*Callorhinus ursinus*) possiblement en lien avec une période de lactation plus longue (3 mois) (Cavagnolo and Vedros, 1979). Chez l'éléphant de mer boréal une augmentation rapide des niveaux d'IgG, d'une sous-classe d'IgG (H24b) et d'une protéine semblable à l'IgM dans le sérum des nouveau-nés était observée durant la lactation (King et al., 1998). Chez les phoques gris, la prolifération lymphocytaire était stable chez des jeunes âgés de 2 à 3 semaines, puis elle augmentait significativement et atteignait un plateau une semaine plus tard, tandis que la phagocytose diminuait chez des jeunes âgés entre 2 et 5 semaines, puis atteignait un plateau (Lalancette et al., 2003). L'ensemble de ces données supporte l'idée que les nouveau-nés de mammifères marins naissent avec un système immunitaire fonctionnel prêt à répondre aux pathogènes qu'ils vont rencontrer dans leur environnement.

1.3. La dynamique des contaminants chez les phoques

L'analyse des concentrations de contaminants dans différents tissus biologiques issus des phoques permet aujourd'hui de mieux connaître la contamination présente dans leur environnement. Cependant, il est nécessaire de mieux définir le transfert des polluants organiques persistants (POP) durant la lactation chez les phoques, de mieux comprendre les facteurs régissant la répartition géographique des contaminants et enfin d'évaluer le transfert des contaminants à travers les réseaux trophiques.

Les contaminants adsorbés ou liés aux matières particulaires présentes dans la colonne d'eau sont rapidement absorbés par le plancton et par d'autres organismes filtreurs; ils sont alors accumulés et concentrés dans les tissus adipeux (par exemple, les composés organochlorés ou organobromés) ou dans les tissus riches en protéines (comme dans le cas des métaux). Un grand nombre de ces contaminants vont persister dans les tissus, de sorte que les prédateurs supérieurs tels que les phoques vont accumuler tout au long de leur vie les charges en contaminants de leurs proies

(contamination trophique). Il arrive cependant que les animaux soient exposés à certains contaminants, ils ne s'accumulent pas dans l'organisme, comme c'est le cas pour les HAP. Il existe une distinction entre l'accumulation de contaminants chez les mâles et les femelles. Ainsi, les mâles vont avoir une accumulation constante tout au long de leur vie, alors que chez les femelles les contaminants vont s'accumuler graduellement dans leurs réserves lipidiques (lard) jusqu'à ce qu'elles donnent naissance et élèvent leur premier chiot (Helle et al., 1976). Ainsi, l'accumulation chez les femelles est entrecoupée par des périodes de détoxification se produisant lors des gestations et des périodes de lactation (Addison and Brodie, 1987) (cf. figure 2).

Chez les mammifères, la lactation est la composante de la reproduction la plus coûteuse en énergie (Gittleman and Thompson, 1988) et représente une portion significative du budget énergétique d'une femelle adulte mammifère (Brody and Lardy, 1946). C'est aussi la période où les femelles phoques vont produire un lait (très) riche en lipides (50% chez le phoque commun, 60% chez le phoque gris et 57% chez le phoque du Groenland) (Boness and Bowen, 1996). L'énergie contenue dans le lait est absorbée très efficacement par les chiots (49% par les chiots de phoque commun, 54-57% par les chiots de phoque gris et 77% par les chiots de phoque du Groenland) (Stewart and Lavigne, 1984; Anderson and Fedak, 1987; Bowen et al., 1992a). La demande énergétique pour produire le lait est très élevée, entrainant une diminution de la masse corporelle des mères. Ainsi, pendant la lactation, les femelles phoque commun, qui se nourrissent durant cette période, vont perdre en moyenne 1,4 kg/jour (Bowen et al., 2001). Les femelles phoque gris, qui jeunent durant la lactation, vont réduire leur masse corporelle de 3,8 kg/jour (Anderson and Fedak, 1987), tandis que les femelles phoque du Groenland, qui jeunent également durant la lactation, vont perdre 3.1 kg/jour (Kovacs et al., 1991). Il en résulte que pour répondre au besoin énergétique de la fabrication du lait, des lipides issus du lard vont être remis en suspension, et par conséquent les contaminants lipophiles préalablement stockés dans le lard vont être aussi intégré dans le processus de production du lait. Par exemple, chez le phoque gris, une femelle peut réduire sa charge en organochlorés de 15-30% durant les 15-20 jours que dure la lactation (Addison and Brodie, 1977), et au sevrage environ 98% des résidus du jeune sont accumulés depuis le lait maternel (Addison and Stobo, 1993). Ainsi, bien qu'un transfert de contaminants se produise durant la gestation (Tanabe et al., 1982), c'est pendant la lactation que l'accumulation maximale se produit (Debier et al., 2003).

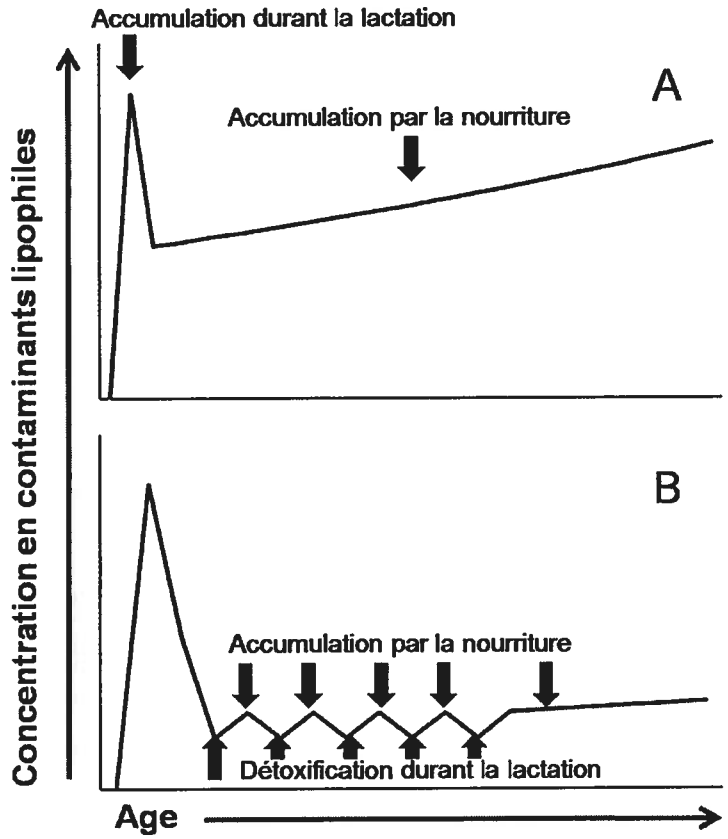

Figure 2. Modèle graphique des différences entre les mâles (A) et les femelles (B) dans la dynamique d'accumulation des composés lipophiles en fonction de l'âge (inspiré de Hickie et al., 2000).

1.3.1. Les organoétains dans les organismes marins

Voir le chapitre 2 de la thèse (section 2.2.4 Organotins in marine organisms).

1.3.2. Charges corporelles en PBDE chez les mammifères marins dans l'hémisphère Nord

Les polybromés diphényles éthers (PBDE) sont des composés chimiques anthropogéniques intensivement utilisés comme retardateurs de flammes dans de nombreux produits plastiques et textiles (résumé par De Wit, 2002). Les PBDE sont vendus sous trois formules : Penta-, Octa- et Déca-BDE. Les deux premières ont été retirées du commerce en Europe (1998) puis en Amérique du Nord (2004), puisque les PBDE qu'elles contenaient étaient considérés comme persistants, bio-accumulatifs,

toxiques et transportés sur de longues distances. Le Déca-BDE dont le principal congénère (à 90 %) est le BDE-209 sera interdit en Europe mais reste actuellement sur les marchés canadiens et américains.

Les PBDE, comme tous les POP, sont persistants, lipophiles et leur solubilité dans l'eau est faible. Ils sont structurellement reliés aux polychlorodiphényles éthers, PCDE, aux polybromés biphényles, PBB et aux biphényles polyclorés, BPC (Figure 3). Les PBDE se retrouvent dans l'environnement marin où ils se concentrent à travers la chaîne alimentaire (Stapleton and Baker, 2003). Les concentrations de PBDE suivent un gradient d'urbanisation et/ou d'industrialisation, comme démontré dans la figure 4. Leurs caractéristiques font qu'ils se dispersent dans le monde de façon similaire aux BPC. Leurs concentrations sont en augmentation depuis ces dix dernières années dans des régions isolées telle que l'arctique, (Malmquist et al., 2003), où ils se biomagnifient à travers le réseau trophique (Wolkers et al., 2004; Sormo et al., 2006; Kelly et al., 2008). Les concentrations de PBDE ont augmenté exponentiellement dans le lard de phoques annelés des territoires du Nord-Ouest sur la période allant de 1981 à 2000 (Ikonomou et al., 2002). Cependant, les vitesses d'accumulation diffèrent entre les groupes de PBDE. Ainsi, les concentrations de penta- et hexa-BDE ont doublé dans le lard des phoques annelés en l'espace de 4,7 années et 4,3 années, respectivement, tandis que, les concentrations de tétra-BDE ont doublé en l'espace de 8,6 années (Ikonomou et al., 2002). Les concentrations des congénères de PBDE les plus dominants (PBDE-47, -99, -100 et –154) dans le lard des bélugas mâles du sud-est des îles Baffin ont augmenté d'un facteur allant de 6,5 à 30,6 entre 1982 et 1997 (Stern and Ikonomou, 2000). De plus, les analyses d'échantillons biologiques (lait, sang et tissus humains) indiquent que les concentrations de PBDE sont à la hausse en Amérique du Nord (Hites, 2004), et des données canadiennes montrent que les fortes concentrations de PBDE mesurées dans le lait humain approchent ou dépassent celle des PCB (She et al., 2007). Des prévisions suggèrent que les PBDE surpasseront les BPC dans de nombreuses populations de poissons et mammifères marins dans les 10 ans à venir (Ikonomou et al., 2002; Ross, 2006). Lebeuf et al. (2004) démontrent une augmentation récente et importante des niveaux de PBDE dans les bélugas du St-Laurent.

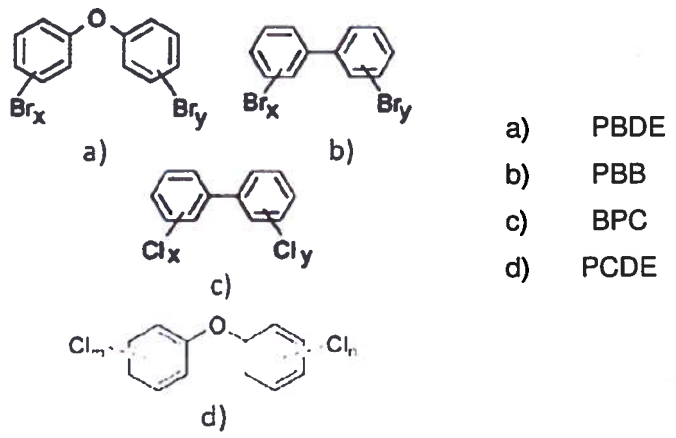

Figure 3. Structures chimiques des composés.

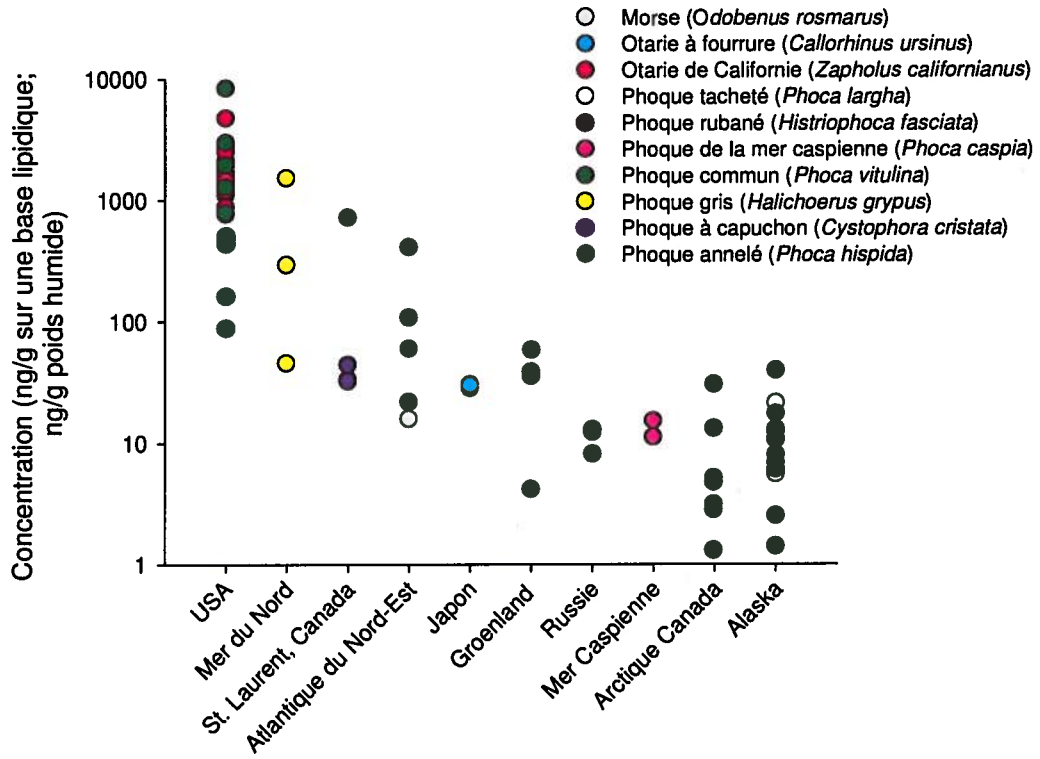

Figure 4. Concentrations de PBDE mesurées dans le gras de différentes espèces de pinnipèdes échantillonnées entre 1997 et 2003 provenant de plusieurs régions dans le monde. Les données proviennent de She et al., 2002; Law et al., 2003; Kajiwara et al., 2004; Kalantzi et al., 2005; Stapleton et al., 2006; Wolkers et al., 2006; Quakenbush, 2007.

1.3.3. Charges corporelles en BPC chez les mammifères marins de l'hémisphère Nord

Il a été démontré que les BPC se bio-accumulent dans les tissus de tous les mammifères marins, incluant ceux des régions isolées telles que l'Arctique (Mössner and Ballschmiter, 1997). Cependant, de façon générale, les mammifères marins de la zone tempérée de l'hémisphère Nord, particulièrement des espèces piscivores des latitudes moyennes d'Europe et d'Amérique du Nord, montrent les plus grandes charges en BPC (résumé par Aguilar et al., 2002). Ces résultats sont consistants avec les études réalisées dans les années 80 portant sur la distribution géographique des organochlorés dans l'atmosphère et les eaux de surface (Tanabe et al., 1983) et avec les patrons précédemment observés dans les tissus de mammifères marins (O'Shea and Brownell, 1994; Reijnders and de Ruiter-Dijkman, 1995) et sont reliés à la production intensive et l'utilisation d'organochlorés dans les pays industrialisés dans ces parties du monde (Reijnders and de Ruiter-Dijkman, 1995).

Les concentrations les plus fortes de BPC sont retrouvées dans les populations de la Mer Méditerranée, comme chez le dauphin bleu et blanc (*Stenella coeruleoalba*) et le dauphin commun (*Delphinus delphis*) (Aguilar et al., 2002). La côte Ouest des États-Unis, particulièrement la Californie, montre des résultats similaires ou légèrement plus faibles. Ces concentrations sont reliées à la densité humaine et les activités industrielles et agricoles intenses de ces deux zones. Bien que moins polluées que les zones précédemment citées, d'autres zones fortement urbanisées et/ou industrialisées dans l'hémisphère Nord montrent aussi de fortes concentrations de BPC, telles que les régions tempérées est et ouest de l'Atlantique Nord, la mer du Nord et les eaux autour du Japon (résumé par Aguilar et al., 2002).

1.3.4. Contaminants métalliques et santé des mammifères marins

Les concentrations en métaux chez les mammifères marins et leurs effets connus ont été résumés par Das et al. (2003). Bien qu'il existe de nombreuses études sur les charges corporelles en métaux chez les mammifères marins, les effets de ces contaminants sur la santé des mammifères aquatiques sont peu documentés. Hyvärinen et Sipilä (1984) trouvèrent une relation entre les cas de mort-nés chez le phoque annelé (*Pusa hispida saimensis*) et les concentrations de nickel (Ni) dans les échantillons de

poils. Dans une étude expérimentale, la léthargie, la perte de poids et finalement la mort étaient observées chez le phoque du Groenland suite à l'ingestion journalière de methylmercure (25 mg/kg de poids corporel/jour) (Ronald et al., 1977). Les paramètres sanguins correspondant indiquaient une défaillance rénale, une urémie et une hépatite toxique. Cependant, cette étude expérimentale ne reproduisait pas l'apport réel de methymercure par la nourriture. En effet, Nigro et Leonzio (1993) calculèrent chez les dauphins vivant en Méditerranée que l'apport journalier de mercure pour un adulte était seulement de 0.9 mg/kg de poids corporel/jour. Une autre étude démontra chez des grands dauphins échoués qu'une inhibition de l'activité des enzymes digestives induite par le mercure provoqua une accumulation de lipofuscine dans les cellules hépathiques, ce qui résulta finalement dans un nombre croissant de maladies hépatiques (Rawson et al., 1993). Des cas de toxicose au plomb ont été démontrés chez le grand dauphin (Shlosberg et al., 1997) et le phoque commun (Zabka et al., 2006). Des études sur les stéroidogenèses surrénalienne et sexuelle chez le phoque gris et le phoque du Groenland indiquèrent que les biosynthèses pouvaient être altérées par les métaux (Freeman et al., 1975; Freeman and Sangalang, 1977). De plus, des phoques du Groenland intoxiqués au methylmercure présentaient des dommages à un faible niveau des cellules sensorielles de l'organe de Corti (Ramprashad and Ronald, 1977).

Dans certaines études, une approche indirecte a permis d'estimer l'implication potentielle des métaux dans une plus faible résistance aux maladies. Des tumeurs sont fréquemment diagnostiquées chez les bélugas vivant dans les eaux polluées de l'Estuaire du St. Laurent (Martineau et al., 2002). Ces animaux présentent de fortes concentrations d'organochlorés, de métaux lourds et de benzo[a]pyrene (Wagemann et al., 1990; De Guise et al., 1994). De Guise et al. (1994) ont suggérés que les contaminants pourraient avoir des effets carcinogéniques et/ou diminuer la résistance au développement des tumeurs. Siebert et al. (1999) ont établit un lien entre les charges corporelles en mercure et les maladies chez les marsouins de la Mer du Nord et de la Mer Baltique. De plus, des niveaux croissants de zinc ont été corrélés avec une détérioration de l'état de santé des marsouins de la Mer du Nord (Das et al., 2004). Enfin, une étude post-mortem sur des marsouins des côtes britanniques a révélé qu'une prévalence de maladies infectieuses est associée avec de fortes concentrations de mercure, de sélénium et de zinc (Bennett et al., 2001).

1.3.5. HAP et santé des mammifères marins

En dépit des preuves de l'exposition des mammifères marins aux HAP et leur potentielle influence sur la santé, ces contaminants ont reçu peu d'attention pour la recherche chez les mammifères marins (Martineau et al., 1994; Fossi et al., 1997a).

Le 24 mars 1989, le superpétrolier Exxon Valdez s'échoue dans le détroit du Prince William, en Alaska. L'échouement provoque le déversement de plus de 40 000 tonnes de pétrole brut dans l'océan. Les études conduites dans le cadre du programme « Natural Resources Damage Assessment » documentèrent un impact substantiel du déversement sur les phoques communs (résumé dans Loughlin, 1994). Approximativement 300 phoques sont estimés être morts suite au déversement, et le nombre de nouveau-né en 1989 était environ 26% plus faible que la normale (Frost et al., 1994). De plus, des lésions oculaires, une irritation de la peau, et des lésions au foie ont été rapportées chez 27 phoques communs échantillonnés suite au déversement de l'Exxon Valdez (Spraker et al., 1994). Les dommages les plus néfastes étaient sur les nerfs du cerveau (Spraker et al., 1994). La toxicité aux hydrocarbures résultait dans des lésions au cerveau dans le thalamus, ce qui pourrait avoir conduit à des problèmes de thermorégulation dus à l'incapacité de déterminer et de contrôler les changements de température. De plus, ces lésions au cerveau pourraient expliquer chez les phoques affectés leurs difficultés à réaliser des activités normales telles que la nage, l'alimentation et la plongée (Lowry et al., 1994). Cependant certains effets observés chez ces animaux sont controversés. Ainsi, chez les 18 nouveaux-nés de phoque commun ayant subi ce déversement et ayant été réhabilités et relâchés, les valeurs de trois paramètres biochimiques (l'aspartate aminotransférase, l'alanine aminotransférase et la lactate deshydrogénase) étaient anormalement élevées, et deux d'entre-eux montrèrent une diminution marquée dans le volume cellulaire. Williams et al. (1994) conclurent que des facteurs autres que le pétrole pourraient avoir été responsables de ces changements, comme par exemple le stress lié à la capture, la manipulation ou le transport.

Les réponses biochimiques aux composés xénobiotiques furent utilisées pour estimer la santé de populations menacées de pinnipèdes, telles que les lions de mer (*Otaria flavescens*) en Amérique du Sud. Une colonie vivant dans un environnement pollué (Mar del Plata, Argentina) et une colonie vivant dans un environnement témoin (Punta Bermeja, Patagonia, Argentina) furent étudiées dans Fossi et al. (1997a). Les

différences dans l'activité des réponses biochimiques aux composés xénobiotiques étaient probablement dues aux différents niveaux de HAP. En effet les quatre HAP les plus cancérigènes retrouvés dans les échantillons de peau étaient cinq fois plus élevés dans la colonie de Mar del Plata (Fossi et al., 1997b).

Le taux de cancers dans la population des bélugas du St.-Laurent est plus élevé que dans n'importe quelle autre population d'animaux aquatiques ou terrestres (Martineau et al., 2002). Or, des produits cancérigènes sont présents dans l'environnement des bélugas de l'Estuaire du St.-Laurent et sont possiblement ingérés par ces animaux. En effet des concentrations élevées de HAP sont retrouvées aussi bien dans le sédiment (celui de la Rivière Saguenay contient de 500-4500 ppb de HAP provenant des alumineries suitées en amont) (Martel et al., 1986) que dans les animaux (les moules bleues situées à l'embouchure de la Rivière Saguenay contenaient trois fois plus de HAP que celles provenant de l'Estuaire ou du Golfe du St.-Laurent) (Cossa et al., 1983). De plus, les invertébrés vivants dans le sédiment contaminé par les HAP accumulent ces composés, contrairement aux vertébrés (Ferguson and Chandler, 1998). Or, les bélugas creusent dans le sédiment (Dalcourt et al., 1992) et se nourrissent d'invertébrés benthiques dans l'Estuaire du St.-Laurent (Vladykov, 1946). Probablement à cause de cette diète et de ce comportement fouisseur, des adduits de l'ADN du B[a]P (voir section 1.7.3.) ont été détectés dans les tissus de bélugas échoués provenant de l'Estuaire du St.-Laurent mais pas dans ceux provenant de l'arctique, lesquels sont supposés vivre dans un environnement moins pollué (Martineau et al., 1988; Shugart et al., 1990). Ces observations suggèrent que le béluga de l'Estuaire du St.-Laurent ingère les HAP présents dans les invertébrés benthiques, ce qui pourrait contribuer au taux élevé de cancer du tractus digestif reporté dans cette population (Martineau et al., 1988; De Guise et al., 1994; Martineau et al., 1994). Cette relation avait été préalablement mise en évidence entre l'ingestion de nourriture contenant du B[a]P et des adénocarcinomes de l'intestin chez la souris (Culp et al., 1998) et des cancers de l'intestin grêle chez l'humain (Chow et al., 1993).

1.4. L'immunotoxicologie (et la génotoxicologie) chez les mammifères marins

Certains contaminants, tels que les BPC, ont fait l'objet de nombreuses études immunotoxicologiques chez les phoques (étude *in vitro*). Cependant, les effets

immunotoxiques de nombreuses autres familles de contaminants (Butylétains, PBDE, certains métaux lourds et les HAP) sont de nos jours encore peu étudiés.

1.4.1. L'immunotoxicologie chez les mammifères marins

Certains contaminants et/ou leurs produits de dégradation (issus de leur biotransformation) peuvent avoir un impact direct ou indirect sur le fonctionnement du système immunitaire. L'impact peut aussi bien se situer au niveau de l'organe, de la cellule ou même au niveau d'une fonction immunitaire spécifique. Les mammifères marins qui sont des organismes fortement contaminés par les polluants organiques persistants (POP), dû à leur position trophique élevée, sont les plus vulnérables à des effets néfastes sur la santé.

Les études immunotoxicologiques prennent en considération les altérations immunitaires stimulatrices ou suppressives, leurs mécanismes et les effets en résultant sur la susceptibilité aux ou à la durée des infections, allergies ou maladies autoimmunes (Burleson and Dean, 1995).

Par le passé, certaines études ont pu exposer *in vivo* des mammifères marins (phoques communs) à des contaminants environnementaux et mesurer leurs effets chez ces animaux (De Swart et al., 1994; Ross et al., 1995; Ross et al., 1996a). Aujourd'hui, pour des raisons d'éthique, de logistique et économique, les recherches sur l'immunotoxicologie chez les mammifères marins se fait à travers des expositions in vitro visant à estimer la toxicité d'un contaminant ou d'un mélange de contaminant sur les réponses cellulaires du système immunitaire. Cependant, cette méthode a des limites et ne permet pas, par exemple, d'évaluer l'absorption du composé, son métabolisme et son excrétion. Un modèle animal, comme le rongeur, peut alors fournir une étape intermédiaire pour l'estimation de la toxicité.

Il est important de noter que chez les phoques, les chiots durant la lactation ingèrent une partie significative de la charge de contaminants de leurs mères via le lait au moment où ils sont dans des périodes critiques de développement du système immunitaire et neurologique (Hall et al., 2003b) et sont particulièrement sensibles aux effets nuisibles des contaminants lipophiles, même à de faibles niveaux d'exposition. Les effets potentiels incluent des désordres des fonctions immunitaires menant à l'augmentation de la susceptibilité aux infections et augmentent indirectement la mortalité post-sevrage. La variation dans la sensibilité aux effets toxiques est dépendante non seulement de l'âge du développement mais aussi du genre, de

l'espèce, de la dose, de la voie d'exposition et des congénères produits (McConnell, 1985; Tilson et al., 1990).

1.4.2. Organoétains et toxicité

Voir le chapitre 2 de la thèse (section 2.3 Toxicity to marine organisms).

1.4.3. PBDE et toxicité

Des études en laboratoire ont démontré que la plupart des congénères de PBDE pouvaient être hépatoxique (Darnerud et al. 2001), immunotoxique et agir comme perturbateurs endocriniens (Byrne et al. 1987). Ils sont actuellement soupçonnés d'être des perturbateurs endocriniens chez les mammifères marins (Darnerud, 2008), notamment chez les cétacés (Fossi et al., 2006) et les chiots de phoques gris (Hall et al., 2003). Une étude démontre que l'atrophie du thymus et la déplétion de la rate chez le marsouin (*Phocoena phocoena)* étaient significativement corrélées à l'augmentation des niveaux de PBDE chez ces animaux (Beineke et al., 2005). McKinney et al. (2006) ont démontré la biotransformation de ces composés chez le béluga pouvant suggérer la formation de sous-produits toxiques.

1.4.4. BPC et santé des mammifères marins

De nombreux rapports indiquent que les BPC peuvent affecter les mammifères marins. Ils furent longtemps soupçonnés d'avoir joué un rôle dans les nombreuses épizooties encourues par les mammifères marins ces dernières décennies. Par exemple, les concentrations élevées de BPC dans les phoques du Lac Baïkal (jusqu'à 64 µg/g total) suggèrent que les victimes de l'épizootie pourraient avoir eu de fortes concentrations de composés potentiellement immunotoxiques (Nakata et al., 1995). De plus, une comparaison des concentrations de contaminants chez les marsouins du Royaume-Uni dont certains mourraient de traumas ou de maladies infectieuses révéla que les niveaux de BPC étaient de 2 à 3 fois plus élevés chez ceux qui succombaient aux maladies (Jepson et al., 1999). Hall et al. (1992) établirent que lors de l'épizootie de

1988 les phoques commun avec de plus fortes charges d'organochlorés étaient plus susceptibles à l'infection au morbillivirus (phocine distemper virus ou PDV) que ceux avec des charges plus faibles. Enfin, l'infection virale et les mortalités de masses du grand dauphin aux États-Unis (Kuehl et al., 1991) et du dauphin bleu et blanc de Méditerranée (Kannan et al., 1993) ont été associées avec la présence de grandes concentrations de BPC dans les tissus des individus affectés.

Un lien a également été établi entre un succès reproductif moindre et la pollution aux BPC (Reijnders, 1980). Ainsi, des sténoses et des occlusions utérines ont été reportées chez différentes populations de phoques en lien avec la charge de BPC (Helle et al., 1976a, b; Baker, 1989). De plus, des naissances prématurées ont été associées chez l'otarie à fourrure à de fortes concentrations d'organochlorés (Delong et al., 1973). Les marsouins de Dall du Pacifique du Nord-Ouest présentaient de faibles taux de testostérone en relation avec les concentrations en BPC (Subramanian et al., 1987). Enfin, des femelles phoque commun nourries avec des poissons pollués avaient un plus faible succès reproductif (50%) lorsque comparés à des femelles nourries avec des poissons moins pollués (Reijnders, 1986). Des tumeurs et un faible taux de reproduction chez les bélugas du St-Laurent pourraient également être lié aux fortes concentrations de BPC retrouvées chez ces animaux (Martineau et al., 1994; De Guise et al., 1995).

Il existe également un lien entre les BPC et un impact sur le système endocrinien. Lors de l'épizootie au morbillivirus en 1988 (le virus découvert chez les phoques était antégéniquement apparenté au Canine Distemper Virus (CDV), responsable de la Maladie de Carré chez le chien, mais distinct, et spécifique du phoque appelé Phocine Distemper Virus ou PDV), des lésions histologiques (tissu colloïde et fibrose) ont été reportés dans les glandes thyroïdes des phoques communs dont les niveaux de BPC étaient fortement élevées comparativement aux niveaux des phoques commun de l'Islande (Schumacher et al., 1993). De plus, il avait été préalablement établi que suite à l'ingestion chronique de poissons contaminés aux BPC, le taux d'hormones thyroïdiennes diminuaient chez les phoques commun (Brouwer et al., 1989).

Enfin, des effets néfastes sur le système immunitaire ont été observés suite à l'exposition aux BPC. Lavhis et al. (1995) ont établit un lien entre les teneurs en BPC et une diminution de la fonction des cellules T chez les grands dauphins capturés dans le Golfe du Mexique. Beineke et al. (2005) ont démontré que l'atrophie du thymus et la déplétion de la rate sont significativement corrélées avec des concentrations croissantes de BPC. Aguilar and Borrell (1994) suggèrent trois explications possibles pour le lien

apparent entre les concentrations élevées de BPC et les maladies et mortalités observées chez le dauphin bleu et blanc: 1) une diminution de l'immunocompétence conduisant à une augmentation de la susceptibilité aux maladies; 2) une mobilisation des réserves de gras conduisant à une augmentation de la teneur sanguine en BPC qui pourrait résulter en une lésion du foie pouvant augmenter la susceptibilité; 3) une existence préalable d'une lésion hépatique spécifique produisant un dysfonctionnement du foie, lequel pourrait résulter à la fois en une l'augmentation des concentrations en BPC et une plus grande susceptibilité.

1.4.5. BPC et immunotoxicité

Une étude *in vitro* visait à mieux comprendre l'implication des contaminants dans l'épizootie à morbillivirus qui toucha les phoques communs de l'Europe du Nord en 1988. Durant une période de deux ans et demi, deux groupes de 11 phoques communs (sept femelles et quatre mâles, dans chaque groupe) ont été nourris avec du hareng, destiné à la consommation humaine, provenant soit de la Mer Baltique fortement polluée, soit de l'Océan Atlantique faiblement pollué. Durant cette période, des changements dans la fonction immunitaire ont été évalués. Les fonctions immunitaires telles que l'activité cytotoxique des cellules NK, la prolifération des cellules T en présence de mitogène, la prolifération spécifique à un antigène et la production d'anticorps étaient diminuées chez les animaux nourris avec les poissons les plus contaminés. Les concentrations de BPC dans les phoques du groupe exposé étaient 2 fois plus élevées que celles du groupe témoin (De Swart et al., 1994; 1995; 1996; Ross et al., 1995; 1996a; 1996b).

Bien que les congénères de BPC testés (BPC 138, 153, 169 ou 180) n'induisaient aucun effet sur la phagocytose, la prolifération des lymphocytes de béluga exposés *in vitro* à 20 ppm ou plus de BPC 138 était significativement réduite lorsque comparé aux cellules témoins, cependant aucune inhibition n'était observé en présence de BPC 153, 180 ou 169 (De Guise et al., 1998). De plus, la prolifération spontanée (sans mitogène) des splénocytes était significativement inhibée par le BPC 138 à 20 ppm ou plus et le BPC 180 à 25 ppm (De Guise et al., 1998). Lorsque les splénocytes étaient exposés à un mélange de 5 ppm de plusieurs congénères de BPC (138, 153 et 180) la capacité des splénocytes à proliférer était significativement réduite, tandis qu'aucun effet n'était observé lorsque les cellules étaient exposées à chaque congénère de BPC individuellement à des concentrations inférieurs à 20 ppm (De Guise et al.,

1998). Ces données suggèrent un effet synergique de certains composés organochlorés sur les splénocytes de bélugas démontrant le danger des mélanges complexes retrouvés dans l'environnement. De plus, le congénère 138 de BPC était le plus immunotoxique (De Guise et al., 1998). Levin et al. (2004) étudièrent l'effet de l'exposition *in vitro* à différents congénères de BPC (138, 153, 169 et 180) à 5 ppm sur la phagocytose chez le grand dauphin et le béluga. Tous les mélanges contenant au moins deux BPC non-coplanaires réduisaient significativement aussi bien la phagocytose des neutrophiles et des monocytes, avec des effets cependant plus marqués chez le grand dauphin que le béluga. Le congénère coplanaire (BPC 169) n'avait aucun effet (Levin et al., 2004). Dans une autre étude *in vitro*, le mélange contenant au moins deux BPC non-coplanaires (concentration finale 5 ppm) augmentait la prolifération des lymphocytes T chez le béluga, le dauphin de Commerson (*Cephalorhynchus commersonii*), le phoque commun, l'otarie à fourrure et le lion de mer de Steller, tandis qu'il inhibait la prolifération des lymphocytes T chez l'orque et le grand dauphin (Mori et al., 2006). Enfin, Mori et al. (2008) établirent *in vitro* que les 4 congénères de BPC (138, 153, 169 et 180) à 5 ppm chacun ne supprimaient pas la prolifération des lymphocytes B chez les mammifères marins, c'est-à-dire qu'aucun effet n'était observé (béluga), ou ils augmentaient la prolifération des lymphocytes B (globicéphale, grand dauphin, phoque commun et l'otarie à fourrure du Nord). Ces différentes études suggèrent que le récepteur AhR (récepteur aryl hydrocarbure) n'est pas l'intermédiaire pour tous les effets immunomodulateurs des BPC et qu'il pourrait exister chez les mammifères marins un récepteur qui joue l'intermédiaire pour la toxicité des BPC non-coplanaires.

Mos et al. (2006) établirent un lien entre les concentrations de BPC chez des phoques commun sauvages et une diminution de la phagocytose, de la fonction lymphocytaire T et une augmentation de la flambée oxydative, suggérant une immunotoxicité associée à ces contaminants. Enfin, une étude établit que l'impact des BPC sur le système immunitaire des phoques diffère selon l'espèce (Hammond et al., 2005), ainsi la phagocytose et la flambée oxydative du phoque commun semble plus touchées par l'exposition *in vitro* aux BPC que celles du phoque gris.

1.4.6. Métaux lourds et Immunotoxicité

Dépendamment du métal, de sa spéciation, de sa concentration et de sa biodisponibilité, et de nombreux autres facteurs, une exposition continue à un métal résultera en un effet immunopotentiateur ou immunosuppresseur. De nombreuses études en laboratoire et épidémiologiques ont démontré les effets immunotoxiques des métaux lourds chez une variété d'espèces (Zelikoff and Thomas, 1998). Cependant l'influence des métaux sur les mammifères marins en relation avec la contamination environnementale a été peu évaluée.

Les cellules immunitaires comme les macrophages peuvent incorporer et stocker les métaux dans des granules. Ceci a été démontré avec le mercure et le sélénium chez le grand dauphin, le dauphin de Risso (*Grampus griseus*) et le lion de mer (*Otaria byronia*) (Nigro and Leonzio, 1996) et avec le titane chez le phoque commun (Kakuschke and Prange, 2007).

Chez de nombreuses espèces de mammifères marins, la capacité immunosuppressive des métaux sur la transformation lymphoblastique et la phagocytose a été mise en évidence. Ainsi, une diminution de la prolifération lymphoblastique, chez les splénocytes et les thymocytes de béluga, a été observée suite à une exposition *in vitro* au cadmium, au mercure ou au methylmercure, mais pas avec le plomb (De Guise et al., 1996). Chez les nouveau-nés de phoque commun, la prolifération lymphoblastique était spécialement inhibée par le béryllium, le plomb, le cadmium et le mercure (Kakuschke et al., 2008). Camara Pellisso et al. (2008) ont observé, chez le grand dauphin, une diminution de la prolifération lymphoblastique suite à une exposition *in vitro* au mercure, au plomb ou au cadmium mais pas à l'aluminium ou au chrome. Le methylmercure inhibait *in vitro* la transformation lymphoblastique aussi bien chez le phoque gris (Lalancette et al., 2003) que chez le phoque commun (Das et al., 2008). Le plomb et le chrome n'inhibaient pas *in vitro* la phagocytose chez le grand dauphin contrairement à l'aluminium, le mercure et le cadmium (Camara Pellisso et al., 2008). Chez de jeunes phoques gris, la phagocytose était affectée par une exposition *in vitro* au methylmercure (Lalancette et al., 2003). Enfin, Pillet et al. (2000) ont démontré chez le phoque gris que l'effet du zinc sur la phagocytose était fonction du sexe.

Outre l'immunosuppression, les métaux peuvent également générer une réponse immunitaire exacerbée (effet immunopotentiateur), qui peut conduire à l'hypersensibilité et à l'autoimmunité. L'exposition chronique à des métaux présents dans l'environnement marin pourrait conduire les mammifères aquatiques à développer des réactions d'hypersensibilités. Un test MELISA® (memory lymphocyte immuno-stimulation assay),

basé sur le fait que les lymphocytes, préalablement sensibilisé à un certain métal (« cellules mémoires »), se transforment en blastes et prolifèrent quand ils sont de nouveau exposés à ce métal, a été utilisé avec les phoques de la Mer du Nord (Kakuschke et al., 2005; Kakuschke et al., 2006). Les métaux sensibilisants étaient dans l'ordre Mobyldène, titane > nickel, chrome > aluminium, plomb, béryllium. De plus, une relation entre les niveaux sanguins de métaux et ce dysfonctionnement immunologique a été rapportée (Kakuschke et al., 2005).

1.4.7. Métaux lourds et génotoxicité

Le potentiel génotoxique des métaux a fait l'objet de quelques études chez les mammifères marins. Le methylmercure induisait *in vitro* chez les leucocytes de grand dauphin des cassures simple brin de l'ADN (Betti and Nigro, 1996; Taddei et al., 2001), cependant ces cellules faisaient preuve d'une réparation efficace de l'ADN par la suite (Taddei et al., 2001). De plus, il a été montré que le mercure mais également le methylmercure induisaient la formation de micronucleis dans les fibroblastes de béluga à des concentrations comparables à celles retrouvées dans les tissus de ces animaux (Gauthier et al., 1998).

1.4.8. Hydrocarbures Aromatiques Polycycliques (HAP) et immunotoxicité

De nombreux rapports ont documenté les effets suppresseurs spécifiques des HAP cancérigènes aussi bien sur l'immunité humorale qu'à médiation cellulaire chez les rongeurs et l'humain (Ladics et al., 1991; Ladics et al., 1992; résumé par White et al., 1994; Davila et al., 1996; Salas and Burchiel, 1998). Les effets incluent une diminution de la réponse proliférative des cellules T, la mort des cellules lymphoïdes, l'atrophie des organes lymphoïdes, l'apoptose, la diminution des réponses des cellules B aux mitogènes et antigènes, l'altération de la régulation des cytokines et des changements dans la fonction immunitaire innée.

Neale et al. (2002) ont démontré *in vitro* que le benzo[a]pyrene (B[a]P) à 10 µM inhibait la prolifération des cellules T du phoque commun. Dans cette expérience, seul le composé parent était étudié et aucune analyse complémentaire n'a été effectué pour déterminer si le caractère immunosuppresseur était dû au B[a]P ou à l'un de ses

métabolites. De façon générale, les mécanismes de l'immunotoxicité des HAP sont peu compris (White et al., 1994). De nombreux mécanismes ont été proposés, certains impliquant la formation de métabolites réactifs via le cytochrome P450. Les lymphocytes périphériques humains possèdent des cytochromes P450 qui métabolisent les HAP (Mudzinski, 1993). Or, de récents travaux indiquent que plusieurs espèces de mammifères marins possèdent des enzymes hépatiques P450 avec une activité similaire à celles des animaux de laboratoire (Hart et al., 1996; Addison et al., 1998; Stegeman et al., 1998; Teramitsu et al., 2000). Il a donc été supposer que les lymphocytes périphériques des mammifères marins possèdent également des cytochromes P450 métabolisant les HAP (Neale et al., 2002).

Neale et al. (2005) explorèrent les mécanismes régissant *in vitro* les effets immunomodulateurs des HAP sur les leucocytes de phoque commun. L'emphase a été mise sur les altérations dans la transcription du gène de la protéine tyrosine kinase, laquelle joue un rôle critique dans l'activation des cellules T, de l'interleukine-1, une cytokine produite principalement par les macrophages et dont la principale fonction est d'assurer les réponses inflammatoires dans l'immunité innée, et de l'interleukine-2, une cytokine jouant un rôle majeur dans l'initiation des réponses immunitaires adaptatives. Les auteurs démontrèrent que suite à une exposition *in vitro* à 20 µM de B[a]P l'expression des tyrosine kinases Fyn et Itk était significativement augmentée, tandis que l'expression des interleukines 1 et 2 était diminuée. Les auteurs en concluent que l'altération de la fonction lymphocytaire est régie par la perturbation de la signalisation du récepteur des lymphocytes T et la production de cytokine. De plus, la forte suppression de l'expression de l'interleukine-1 suggère un effet inhibiteur direct du B[a]P sur les macrophages.

1.4.9. HAP et génotoxicité

Plusieurs études portant sur l'altération des molécules d'ADN ont été réalisées sur des mammifères marins échoués. L'exposition d'un organisme à un chimique génotoxique pourrait résulter dans la formation de lien covalent avec l'ADN de l'organisme (adduit de l'ADN). La détection des adduits est donc un moyen de démontrer une exposition. Cette approche a été utilisée avec l'ADN du cerveau et du foie de béluga échoué des estuaires du St.-Laurent (zone hautement contaminée) et du Mackenzie dans Arctique (zone contrôle) pour déterminer l'exposition au B[a]P (Shugart et al.,

1990). Les adduits qui se forment entre l'ADN et la forme cancérigène du B[a]P étaient détectables dans les bélugas du St.-Laurent mais pas dans ceux de l'Estuaire du Mackenzie. De plus, Martineau et al. (1994) ont détectés des adduits de l'ADN résultant de l'exposition spécifique au B[a]P dans huit des neuf bélugas de l'Estuaire du St.-Laurent échoués durant la période 1983-1990.

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2. TOXICOLOGIE DES ORGANOÉTAINS DANS LES ORGANISMES MARINS : UN RÉSUMÉ

Héloïse Frouin, Émilien Pelletier, Michel Lebeuf, Richard Saint-Louis et Michel Fournier. Toxicology of Organotins in Marine Organisms: A Review. Dans H.F. Chin (Ed), Organometallic compounds : Preparation, Structure and Properties (ISBN : 978-1-60741-917-4). Nova Publishers. (Accepté le 1er avril 2009).

Mon rôle a consisté à rechercher les données déjà existantes dans la littérature,en faire une synthèse et écrire le chapitre de livre à titre d'auteur principal.

RÉSUMÉ

L'environnement marin à été contaminé par les organoétains, suite à leurs utilisations comme agent actif dans les peintures marines "antisalissures" et comme stabilisateurs de chaleur dans les produits en PVC. Dû à leur vaste utilisation, des études ont été initiées pour mettre à la lumière la contamination environnementale et l'impact des organoétains. Le caractère biocide du tributylétain (TBT) devint un problème environnemental lorsque des organismes commerciallement importants, tels que les huîtres, furent touchés. Suite à ces découvertes, des mesures visant à contrôler l'usage des organoétains, furent adoptées par plusieurs pays. Cependant, à l'échelle mondiale, ces actions ne réduirent pas significativement l'utilisation des organoétains. Bien que les effets toxiques des organoétains chez de nombreux organismes aquatiques soient une menace sérieuse, peu de choses sont connues sur l'accumulation et les implications écotoxicologiques le long des réseaux trophiques. Les précédentes revues sur la toxicité des organoétains chez les organismes marins ciblaient principalement la toxicité aigüe. Cependant, il a été démontré que des concentrations très faibles d'organoétains pouvaient également être toxiques chez les organismes. Au vue des différences entre les propriétés chimiques des organoétains, pouvant se répercuter sur les propriétés d'absorption, de distribution tissulaire et de biodisponibilité chez les animaux, cette revue considérera principalement le TBT et ses produits de dégradation, le dibutylétain (DBT) et le monobutylétain (MBT). L'objectif de ce chapitre est de passer en revue les effets de ces organoétains à de faibles concentrations chez plusieurs espèces marines, allant de l'algue au mammifère. De plus, une emphase est portée sur les mécanismes d'actions de ces composés organométalliques.

ABSTRACT

Organotin compounds have entered in the marine environment because of their use as antifouling agents in marine paints and as heat stabilizers in vinyl chloride polymers. Widespread usage of organotins led to a number of studies aimed at shedding light on environmental contamination and its impacts. Biocidal behaviour of tribultytin (TBT) became an environmental issue when the threat to non-target organisms, particularly commercially important bivalves such as oysters became apparent. As a result of these investigations, control measures on the usage of organotins were adopted in several countries. However, such actions did not appreciably reduce the consumption of organotins on a global scale. Even though there is serious concern for the toxic effects of organotin in various aquatic organisms, little is known about the accumulation and the ecotoxicological implications along food chains. Reviews on the toxicity of organotins to marine organisms included a high proportion of acute toxicity data. However, evidence was presented showing that extremely low concentrations of organotins were toxic to some organisms. In view of the differing chemical properties of organotins, which could have different absorption properties and, presumably, tissue distribution and bioavailability in animals, this review will mainly consider TBT and its major metabolites dibutyltin (DBT) and monobutyltin (MBT). The objective of this chapter is to review the effects of these organotins at low concentrations on several marine species, from algae to mammal. Furthermore, an emphasis is placed on the mechanisms of actions of these organometallic compounds.

2.1. Introduction

The organotin compounds contain covalent bonds between tin (Sn) and carbon (C) and have the general formula R_nSnX_{4-n}, in which R is an aryl or alkyl group and X is an inorganic species and n = 1-4. The number of Sn-C bonds and the length of the alkyl chain have a profound effect on the physical and chemical properties of organotins. In general, the water solubility of the compounds decreases as the number and length of the organic substitutes increase, but it also depends on the inorganic substitute, X (Hoch, 2001). Biological degradation, resulting in sequential dealkylation, is the main pathway, although (UV) photodegradation via the same mechanism can be important under some circumstances: $R_4Sn \rightarrow R_3SnX \rightarrow R_2SnX_2 \rightarrow RSnX_3 \rightarrow SnX_4$. In general, the solubility of organotin compounds in water decreases with the increasing number and length of the organic substitutes but it also depends on the particular X.

Organotin compounds have developed into important industrial commodities in the past 50 years because of the wide variety of their uses. About 70% of the organic application (Hoch, 2001) of the world annual production of organotin compounds, estimated at 50,000 tons (Fent, 1996) is the use of mono/dialkyltin derivatives as heat and light stabilizer additives in PVC processing. Major organotin compounds released to the environment are those of triphenyltin (TPT), tricyclohexyltin (TCT), di-n-octyltin (DOT), di-n-butyltin (DBT), dimethyltin (DMT), and tri-n-butyltin (TBT) (Omae, 2003). TPT and TCT species are agricultural pesticides. DOT compounds are used as stabilizers in some food wrappings. DBT and monobutyltin (MBT) are the metabolites of TBT, but they have been produced and used as stabilizers for chlorinated polymers and as catalysts for polyurethane foams and silicones. TBT compounds are used as biocides for cooling systems and as antifouling agents for boat and fishnet paints in maritime activities since the early 1960s (Omae, 2003). TBT-based antifouling paints preventing the attachment of algae and invertebrates on ship hulls have been the main source of organotins in coastal waters for over three decades (Batley, 1996; Alzieu, 2000). Today, biocidal uses, such as in antifoulants, agrochemicals and wood preservatives make up about 20% of the total annual production of organotin compounds (Omae, 2003).

When TBT-based paints were first introduced on the market, manufacturers claimed that the active ingredient was readily degraded once released in water by hydrolysis and photolysis, away from the painted surface. That was true on a small scale, but as the rate of TBT release increased to the point it exceeded the degradation rate, TBT was accumulated in non-target organisms. It became evident in the late 1970s that TBT leached from paints affected organisms other than those targeted. The shells of Pacific oysters, Crassostrea gigas, being farmed in France began to exhibit morphological abnormalities; the annual spat fall also declined dramatically (Alzieu and Portman, 1984). Severity of these effects increased with the proximity to yacht marinas, and TBT came under suspicion. Similarly, in the eastern United States, the imposition of male sex characters ('imposex') on female mud snails, *Ilyanassa obsoleta*, was linked to TBT leached from antifouling paints (Smith, 1981). Additional problems arose during the 1980s when, for example, in Scottish sea lochs antifouling paints were used on cages employed in the salmon farming industry (Balls, 1987).

The environmental damage caused by TBT led to some partial bans on its use during the 1980s and 1990s, and from 2003 the International Maritime Organisation (IMO) has issued a worldwide prohibition scheduled to enter into force in September

2008 (see http://www.imo.org). Restrictions of the use of TBT-based antifouling paint on boats with a length less than 25 m were imposed, first in France (1982), later in the United Kingdom (1987). Similar regulations were enforced in 1989-1990 by many other countries like the USA, the Netherlands, Australia, Canada, Japan, and the European Union but not globally (Champ, 2000). Recent monitoring data using biota demonstrate global pollution of coastal areas. Studies mainly from Asian developing countries indicate a similar contamination pattern of harbours and estuaries as in developed countries. Moreover, monitoring data 7-10 years after the implementation of regulations concerning TBT-antifouling paints demonstrate that the decrease was not substantial, as contamination remains at similar levels (Takeuchi et al., 2004). Only a few reports indicate a decrease in marine systems (Hall et al., 2000). In many locations where regulations have been observed, TBT pollution has decreased in seawater and surface sediment over the last 10 years (Fent, 1996). Despite these improvements, some factors still limit a full recovery of heavily contaminated areas, such as the long-term persistence of TBT in sediments, the diffusion and dispersion of TBT from hulls of large vessels, and, more importantly, the lack of control measures in many developing countries. Despite global trends towards regulating organotins, the use of TBT in antifouling paints is expected to continue in some Asian countries in years to come, primarily due to its relatively low cost and high effectiveness as an antifouling agent (Kwok and Leung, 2005). The estimated organotin consumption in China is 7500 tons/year (Cao et al., 2009). The large use of butyltin compounds in various fields has caused widespread occurrence of water pollution in China (Jiang et al., 2001; Zhou et al., 2001). Unfortunately, except that Pesticide Ordinance prohibited the use of TBT paints on vessels less than 25 m in length and on fish cages in Hong Kong in 1992, no further restrictions on organotins have been implemented in China (Morton and Blackmore, 2001). As a consequence, organotin levels found in environmental compartments and in organisms vary strongly between locations (Tselentis and Tzannatos, 2000; Sudaryanto et al., 2002), not only in coastal and shallow waters with heavy ship traffic, but also in the open ocean. Recent studies have shown the expansion of BTs pollution into developing countries (Sudaryanto et al., 2004; Sudaryanto et al., 2005), in the Great Barrier Reef (Negri and Marshall, 2009) and even to the Arctic region (de Brito et al., 2002) and Antarctic (Negri et al., 2004), most oceanic waters, and deep sea ecosystems, with significant BT residues in organisms (Takahashi et al., 1997). Although the risk of food chain biomagnification is not considered as significant for top-level consumers, a

considerable extent of the contamination has been detected along food chains up to the highest trophic levels such as cetaceans, pinnipeds and seabirds (Takahashi et al., 1999; Berge et al., 2004).

TBT is often present at very low concentrations (< 10 ng/L) in coastal waters (Michel and Averty, 1999a; Gui-Bin et al., 2001; Thomas et al., 2001) and subtle effects of this compounds and its degradation products on the immune and endocrine systems of invertebrates have been clearly identified for very low concentrations in seawater (Bouchard et al., 1999; Alzieu, 2000; St Jean et al., 2002). But other organotins, TPT among them, also show strong biocide effects (Horiguchi et al., 1997). The specific objectives of this review chapter are to gather, synthesize and interpret recent data on organotins in marine environments and organisms, to summarize their toxicity for marine organisms and to describe the mechanisms of actions of these pesticides

2.2. Organotins in the marine environment

2.2.1. In Saline Waters

2.2.1.1. Concentrations

Organotin contamination of marine environment is well documented, notably for TBT. In this review, emphasis is placed on recent data acquired after the regulation on TBT-containing antifouling paints in recreational boats came into effect. Measurements in seawater, taken prior to the restriction on TBT antifouling paints (France in 1982; United Kingdom in 1987; United States in 1989, European Community and Canada in 1990) had shown levels ranging between 50 and 500 ng/L in North American and European marinas, exceptionally reaching peak values around 1 µg/l in highly contaminated areas (Alzieu, 1998). One year before the UK ban, TBT concentration in the Wroxham Broad (a shallow lake) was 898 ng/L (Waite et al., 1989).

The highest organotin levels were and are still found in and nearby marinas and ports due to the use of antifouling paints and painting and depainting operations in dry docks. Contamination decreased slowly in countries where TBT antifouling paints have been regulated (Valkirs et al., 1991; Harino et al., 1999). In France, Michel and Averty (Michel and Averty, 1999a) detected considerable variability in the contamination of 237 sampling points on the French coast. They observed that 75% of their measurements exceeded the detection limit of 1 ng TBT/l (= 0.4 ngSn/L) and the overall average for all sampled sites was 4.6 ng TBT/L, with ten stations exceeding 100 ng TBT/l. No

improvement was observed in comparison with a similar survey carried out in 1992. In Brest naval harbour waters from September 1993 to December 1997, the levels of contamination were more or less the same (14-75 ng/L) during the four-year study period (Chaumery and Michel, 2001). Relatively high concentrations of TBT, 10-78 ng/L, were again detected from several marinas and harbours on the coast of the United Kingdom during 1998, 11 years after the legislation was implemented (Thomas et al., 2001). In Japan, elevated TBT levels in marine systems have been associated with activities linked with pleasure and commercial boat ing, or vessel repair facilities and dry docks, but also with the use of antifouling paints on fish nets and cages in aquaculture (Takeuchi et al., 2004). The butyltins are still widely detectable along the Japanese coast, including international ports and unpopulated areas, even 7-9 years after the partial restriction on TBT usage (in 1990) was implemented in Japan. Elevated levels of organotins were reported with TBT levels reaching 160 ng/L (Takeuchi et al., 2004).

In Asian countries where no specific legislation controlling the use of TBT has been adopted, particularly high levels of contamination are still recorded in harbour waters. Taiwan coastal areas have been reported to be highly polluted by organotins with the TBT concentrations in seawater reaching 480 ng/L and concentrations of butyltins (BTs, including MBT, DBT and TBT) ranging from 90 to 1163 ng/L (Meng et al., 2009). The concentrations of organotin compounds varied in the range of 480-1111 ngSn/L in the sea area of Hong Kong in surface seawater sampled during July 2006 and January 2007 (Tang et al., 2008). In another study conducted in the coastal environment of Xiamen, China, the concentrations ranged from 2-114 ngSn/L of butyltins and 0 to 46 ngSn/L of phenyltins dissolved in the surface water and between 10 to 36 ngSn/L of butyltins and 0 to 1.3 ngSn/L of phenyltins dissolved in the bottom water (Wang et al., 2008). This observation was consistent with a previous study indicating that concentrations of total butyltins in the water column of the Saguenay Fjord (Canada) were significantly higher in surface samples (26 to 206 ngSn/L) than in deep samples (7 to 30 ngSn/L) (Viglino et al., 2006). Moreover, much higher levels can occur in the surface microlayer where naturally occurring lipidic macromolecules can attract lipophylic pollutants (Cleary and Stebbing, 1987; Wang et al., 2008). Table 1 summarizes selected recent data for several regions in the world.

Table 1. Organotin compounds (ng Sn/L) in seawater samples collected in several regions in the world

Sampling		Levels of organotins compounds				Reference
Location	Year	MBT	DBT	TBT	TPT	
North American harbours and marinas						
St Lawrence Estuary, Canada	2002	1.3-7.5	0.6-1.5	2.8-7.4		Mamelona and Pelletier 2003
Gulf of Maine, US	1991	<d.l.-4	<d.l.-1.5	<1-11		Larsen et al. 1997
South American harbours and marinas						
Coatal waters, Argentina	-			0-400		Goldberg et al. 2004
Asian and Oceanian harbour and marinas						
North coast of Kyoto, Japan	2003	2.5-23	2.1-13	3.9-27	<0.18-5.6	Ohji et al. 2007
Coastal waters, Japan	1997-1999	<d.l.-17.6	<d.l.-26.3	<d.l.-160		Takeuchi et al. 2004
Coastal waters, Korea	1997-1998	<d.l.-13.4	<d.l.-22.3	<d.l.-7.7	<d.l.-5.9	Shim et al. 2005
Coastal waters, Taiwan	1998-2004	1.3-6.5	0.3-5.6	<d.l.-480	<d.l.	Meng et al. 2009
Xiamen, China	2006	<d.l.-67	<d.l.-49	<d.l.-60	<d.l.-0.87	Wang et al. 2008
Coastal waters, Singapore	2000	<d.l.-1580	<d.l.-3830	430-3200	<d.l.-400	Basheer et al. 2002
European harbours and marinas						
Coastal waters, United Kingdom	1998			1.2-78		Thomas et al. 2001
South west coast, Spain	1993	<d.l.-51	6.8-20	9.1-79		Gomez-Ariza et al. 1998
North west coast, Spain	-	0.8-11.6	0.3-33.7	0.4-196.6		Rodríguez-González et al. 2006
South east coast, France	1998	-	-	<0.015-0.12		Michel and Averty 1999
Coastal waters, Greece	1998-1999	<d.l.-19	<d.l.-159	<d.l.-70		Thomaidis et al. 2007
Coastal waters, Portugal	1999-2000	<3.3-26	<3.0-30	<3.1-29		Díez et al. 2005
Coastal waters, Corsica	1999	<d.l.-39	0.3-107	2.0-200	<d.l.-1.9	Michel et al. 2001

- : not reported; <d.l. : below limit detection

2.2.1.2. Degradation in Seawater

The degradation of the organotin compounds can be caused by chemical hydrolysis, photolysis and microorganisms. Seligman et al. (1996) suggested that microbial degradation is the most important process mediated by microorganisms such as bacteria, algae and fungi (Looser et al., 2000). Other plant species, including macroalgae and eelgrass, *Zostera marina*, may also play a significant role in the degradation of TBT in some shallow-water areas (Francois et al., 1989). Biodegradation is variable and dependent on environmental conditions and composition of micro-organisms (Seligman et al., 1986; Maguire, 1987; Stewart and De Mora, 1990). The degration of dissolved TBT under the action of bacteria or light has already been considered in various experimental studies. The most commonly reported half-lives are between 4 and 19 days (reviewed by Omae, 2006) but in deep sea half-live is in the order of several years (Michel and Averty, 1999b). Hence, data for the half-lives in natural seawater, in fresh water or in pure water in the laboratory vary with the intensity of light, temperature, types or amounts of organisms, flow rates of seawater, types or amounts of ions in seawater, and amounts of the organotin compounds. However, TBT is quickly removed from the water column and adheres to bed sediments because TBT has a specific gravity near 1.2 kg/L at 20℃ (Landm eyer et al., 2004), low solubility less than 10 mg/L at 20℃ and pH 7 (Fent, 1996), and log K_{ow} values near 4.4 at pH 8 (Meador, 2000).

2.2.2. In Sediments

2.2.2.1. Concentrations

Because TBT and TPT are strongly associated with natural sorbents (Burton et al., 2004) and organic carbon (Brändli et al., 2009) and because they are persistent under anoxic conditions (Fent and Hunn, 1991, 1995), they accumulate in marine sediments. The occurrence of organotin compounds in estuarine superficial sediments has been associated to their historical use, mainly related to fishing activities in the contaminated regions (Arambarri et al., 2003; Nogueira et al., 2003). The organotin decrease is not, or to lesser extent, observable in sediments. TBT and TPT are very

persistent in sediments and are stored for years up to a decade or longer (Maguire, 1987; Fent and Hunn, 1991; Viglino et al., 2004). Sediments act as important reservoirs, but desorption of organotins can also occur (Unger et al., 1988). Resuspension by tide cycles, storms or dredging operations can lead to increased organotin levels in the water column. Sediments of the St. Lawrence Estuary with concentrations of butyltins between 3 and 6 ng Sn/g d.w. (Michaud and Pelletier, 2006) are among the lowest contaminated sediments by BTs reported for coastal zones around the world. Taiwanese coastal sediments contained TBT concentrations (l.d.-8547.6 ng/g d.w.)(Lee et al., 2006) higher than most of developed and developing countries such as the USA (0.3-3.6 ng/g d.w.) (Pereira et al., 1999), Canada (<0.2-80 ng/g d.w.) (St.-Jean et al., 1999), the Øresund strait between Denmark and Sweden (1-19 ng/g d.w.) (Strand et al., 2003), Gulf of Oman (<0.01-60 ng/g d.w.) (de Mora et al., 2003), Malaysia (2.8-1100 ng/g d.w.) (Sudaryanto et al., 2004) and Indonesia (0.57-190 ng/g d.w.) (Sudaryanto et al., 2005). However, sediments from the Spanish north-eastern and south eastern coast (124-18722 ng/g d.w.) (Díez et al., 2002), from five river estuaries of Gipuzkoa (50-5480 ng/g d.w.) (Arambarri et al., 2003), from German North Sea and Baltic Sea marinas (80-17000 ng/g d.w.) (Biselli et al., 2000), and from Hong Kong marinas (<l.d.-53000 ng/g d.w.) (Ko et al., 1995) showed higher TBT concentrations than those from Taiwanese coasts. Data on harbours and estuarine sediments in pleasure boat harbours indicate that TBT residues remain high. Sediment levels are high in ports and boat harbours, depending on the boat frequencies, and generally lower in coastal regions and in some developing Asian countries (Fig. 1).

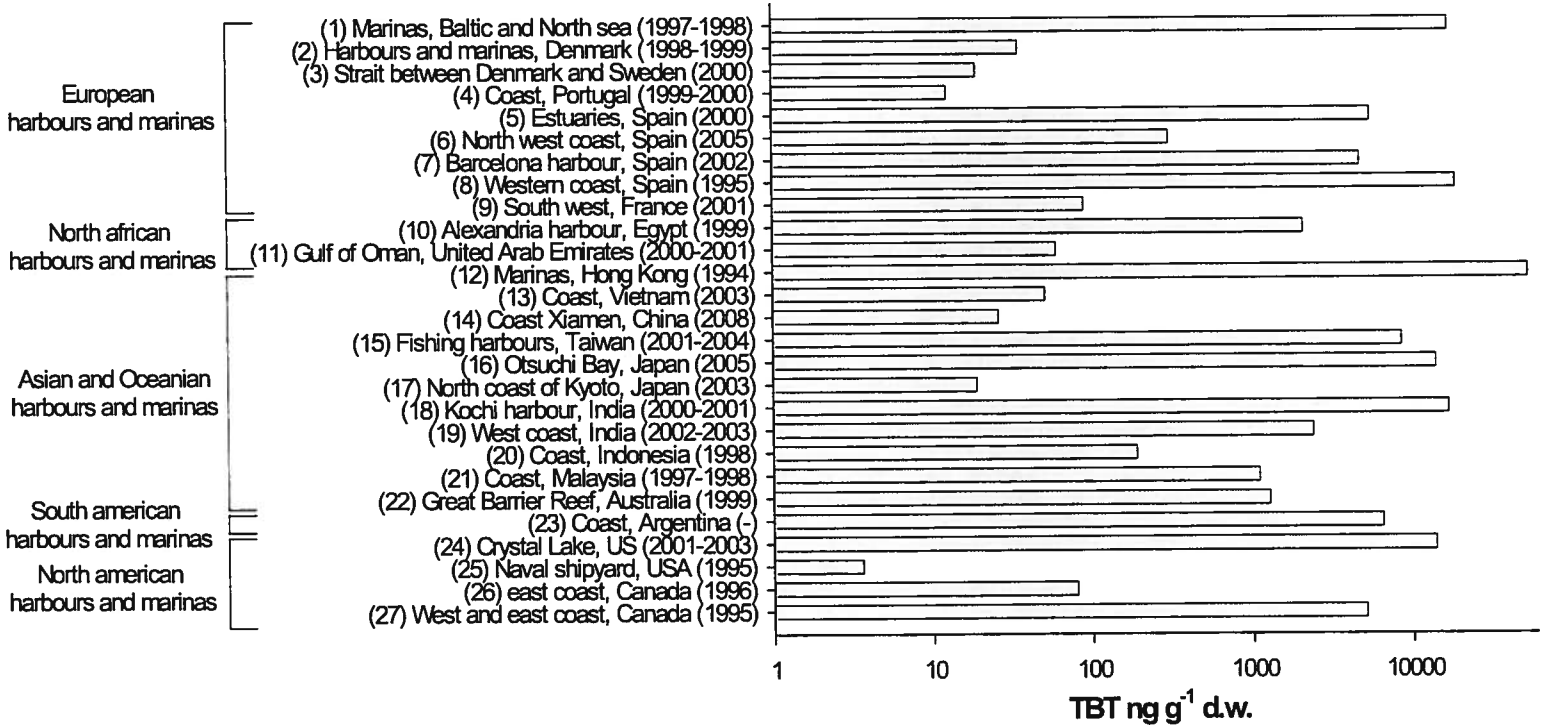
2.2.2.2. Degradation in Sediments

As sediments are the main reservoir for accumulation and storage of organotin compounds, the half-life of TBT in this matrix has often been estimated in the magnitude of 6.9 (Hwang et al., 1999) to 8.7 years (Stewart and Thompson, 1997) in north Pacific coasts. TBT half-life in northern sediments collected in Saguenay Fjord was estimated to be more than 80 years (Viglino et al., 2004). The large discrepancy between reported half-life is clearly related to the activity of the microbial fauna which controls butyltin degradation. The main environmental factors controlling microbial activity are the redox state and temperature of the sediments. Fast degradation was reported in the marine sediment of San Diego Bay (Stang et al., 1992). TBT is often highly persistent in surface

sediment, particularly in cold and anoxic environments because TBT is mainly degraded by aerobic microbial communities strongly affected by low tempertature often observed in deep fjords and bays (Stewart and De Mora, 1990; Chau et al., 1997a; Takahashi et al., 1999).

TBT degradation proceeds through successive dealkylations from TBT to DBT and MBT with intermediate hydroxylated metabolites, and eventually leading to Sn oxides. Furthermore, methylation of organotins can occur in sediments, leading to remobilization to the atmosphere (Amouroux et al., 2000). Some bacteria develop resistance to high levels of organotins in sediments by an adaptation process. They express genes for a multidrug efflux pump and excrete toxic organotins (Jude et al., 2004). As for TBT desorption, it had been established by the mid 1990s that some desorption from estuarine sediments does not occur in parallel with declining water inputs (Langston and Pope, 1995). Desorption has been later confirmed and extended to the less hydrophobic degradation products by different experimental set-ups (e.g. (Dowson et al., 1996; Berg et al., 2001; Burton et al., 2004)) where DBT was more soluble than TBT. Currently, even if it is generally supposed that undisturbed deposits are unlikely to be a significant BT source (Scrimshaw et al., 2005) and an aging effect has been reported for organic-rich sediments (Burton et al., 2006), the disposal of dredged harbour sediments in areas such as the North Sea as before is precluded by their high concentrations (Hamer and Karius, 2005). However, in general, the influence of sediments on the water column concentrations of organotin compounds is insignificant compared to other sources.

Figure 1. Recent data on maximal TBT concentration in marine sediments. TBT concentrations are given for different countries and locations. Years refer to sampling date. Data are from the following sources : (1) (Biselli et al., 2000); (2) (Nogueira et al., 2003); (3) (Strand et al., 2003); (4) (Díez et al., 2005); (5) (Arambarri et al., 2003); (6) (Üveges et al., 2007); (7) (Diez et al., 2006); (8) (Díez et al., 2002); (9) (Bancon-Montigny et al., 2004); (10) (Barakat et al., 2001); (11) (de Mora et al., 2003); (12) (Ko et al., 1995); (13) (Nhan et al., 2005); (14) (Wang et al., 2008); (15) (Lee et al., 2006); (16) (Harino et al., 2007b); (17) (Ohji et al., 2007); (18) (Bhosle et al., 2006); (19) (Bhosle et al., 2004); (20) (Sudaryanto et al., 2005); (21) (Sudaryanto et al., 2004); (22) (Haynes and Loong, 2002); (23) (Goldberg et al., 2004); (24) (Landmeyer et al., 2004); (25) (Pereira et al., 1999); (26) (St.-Jean et al., 1999); (27) (Chau et al., 1997b).



2.2.3. Organotins in marine organisms

2.2.3.1. Introduction

The contamination of marine environment with organotins has spread all over the world in 1980s and 1990s as evidenced by their detection in coastal waters, suspended particulate matter, sediments and particularly biota. The accumulation, transfer and toxicological effects of organotins along the food-chain require attention of environmental toxicologists and a complete characterisation of organotin levels in biological tissues of tinny and large organisms from the lowest and highest trophic levels. Measurements in biota tend to give a better indication of contamination then water samples, because many living organisms can integrate and reflect contamination in their environment over a long period of time. Butyltin and phenyltin concentrations were found in a considerable number of marine organisms and various locations. This hold true in particular for TBT and TPT showing bioaccumulation in biota including mussels (Harino et al., 1999), fish (Lee et al., 2006) and marine mammals (Ciesielski et al., 2004). Because of their filter-feeding behaviour and their high potential for bioaccumulation of toxicants, bivalves have been widely used as sentinel organisms for monitoring organotins (Albalat et al., 2002). Although regulations on butyltin uses in industrial applications have been enforced in the last two decades, coastal waters are still exposed to organotins. As an example, researchers observed that the levels of TBT in North Sea and Baltic Sea organisms remained relatively constant between 1985 and 1999 whereas the TPT concentrations decreased in blue mussels (Rudel et al., 2003). Since the density of marine traffic has not changed significantly during this period, the decline of TPT concentrations seems to be correlated with its use pattern. TPT was no longer used in antifouling paints after 1985 (Bennett, 1996).

2.2.3.2. Bioconcentration and bioaccumulation of organotins

Very few data concerning butyltin contamination in plankton are yet available in the literature. In the St. Lawrence Estuary, BTs concentrations found in zooplankton at the mouth of the Saguenay Fjord (Michaud and Pelletier, 2006) were high (793 ng Sn/g d.w.) and even higher than those measured inside the Fjord by Viglino et al. (2006). In Takahashi et al. (Takahashi et al., 1999), phytoplankton, zooplankton and suspended particulate matter were collected from Otsuchi Bay, Japan, and analysed together and

total BTs up to 1700 ng Sn/g w.w. were found. Most studies concerning the uptake of these pollutants by aquatic organisms deal with TBT because of its extreme toxicity to several organisms. It is shown that some bacteria display a remarkable ability to accumulate this contaminant.

Research on TBT accumulation by aquatic invertebrates has been mostly confined to molluscs (bivalves) and crustaceans (decapods) because these groups are important seafood resources and are ecologically dominant in many habitats. Some studies already demonstrated that butyltins tend to accumulate in digestive organs of invertebrates more than in other organs (Takahashi et al., 1997; Mamelona and Pelletier, 2003). Mussels are known to accumulate large amounts of TBT, especially in viscera and gills (Laughlin et al., 1986; Guo-Lan and Yong, 1995). The lipophilic nature of butyltins and their affinity for membranes are well known (Gray et al., 1987; Powers and Beavis, 1991). The polar seastar, *Leptasterias polaris*, has the capability of eliminating important quantities of TBT and DBT ingested from its preys, and most of the BTs were bioaccumulated preferentially in the pyloric caeca, whereas gonads contained only small quantities (Békri and Pelletier, 2004) and little in the body wall including the calcareous skeleton (Mercier et al., 1994). The studies demonstrated that marine bivalves are able to accumulate significant amounts of TBT (up to > 5 µg/g) (Laughlin, 1996). Crustaceans accumulate much lower amounts of this pollutant because of their ability to efficiently degrade TBT via enzymatic mechanisms in the body (Laughlin, 1996).

Albalat et al. (2002) and Borghi and Porte (2002) found total organotins in flatfish from the Polish coast and in deep-sea fish from the northwestern Mediterranean Sea, respectively, decreased in the following order: liver>digestive tube>gills. Preferential accumulation of BTs and PTs in fish liver has also been reported by many other studies such as Stäb et al. (1996) and Harino et al. (2000). In Lee et al. (2005), fish liver contained the lowest percentage of TBT and the highest percentage of TPT among six organotin compounds, suggesting a certain ability of fish to metabolize TBT in contrast to TPT and a preferential accumulation of TPT in the liver. Among phenyltins, concentrations of TPT were the lowest in the gills and muscles. Moreover, Borghi and Porte (2002) found the highest TPT and TBT in gills and digestive organ of deep-sea fish, and Kannan et al. (Kannan et al., 1996) indicated that TBT was the major butyltin compound representing >60% of total butyltin concentrations in liver, kidney and muscle of blue sharks. Pioneer studies showed the high affinity of trialkyltin compounds to bind to amino acids, peptides and proteins (Davies and Smith, 1980), and their bioavailability

and biotransformation in fish tissues are different. Therefore, this complexe formation between TBT or TPT and metal-binding proteins may influence tissue distribution (Yamada and Takayanagi, 1992; Borghi and Porte, 2002). It has been observed that TBT tends to accumulate more in lipid-rich organs where the order of bioconcentration factors (BCF) was muscle<gill<viscera (Hongxia et al., 1998).

Recent studies have shown that marine mammals and birds also accumulate high levels of toxic butyltins in various tissues and organs (Guruge et al., 1997; Berge et al., 2004). .Although only limited surveys of birds have been undertaken (Guruge et al., 1997; Kannan and Falandysz, 1997), evidence of widespread TBT distribution has nonetheless been obtained. In birds and marine mammals, liver and kidney and some other tissues accumulate organotin significantly more than blubber (Stäb et al., 1996; Tanabe, 1999).

Last decade research interest has focused on marine mammals, especially cetaceans (Kannan et al., 1997a; Kannan et al., 1997b). TBT and its degradation products, DBT and MBT, have been determined in several species of whales and pinnipeds (Tanabe, 1999). Organisms from coastal regions exhibited the greatest organotin accumulation, but measurable quantities were also found in samples from remote locations. In large marine mammals, Iwata et al. (1994) found maximum concentrations reaching up to 770 ng/g w.w. In St. Lawrence Estuary, the liver of beluga whales was contaminated with TBT concentrations reaching up to 467 ng/g d.w. (St.-Louis et al., 2000). Butyltin species tended to be most concentrated within the liver, blubber and muscles of marine mammals. Enormous concentrations of TBT, up to 11,340 ng/g w.w. in the liver, have been reported in bottlenose dolphins found stranded along the US coast, prompting suggesting that this pollutant may have contributed to their demise (Kannan et al., 1997a). The accumulation of TBT by higher trophic aquatic organism proceeds through either uptake from water alone or of a combination with diet ingestion. Significant concentrations were found in various tissues of cetaceans: dolphins (Harino et al., 2008) and whales (Harino et al., 2007a) found stranded along the Thailand coasts, Ganges River dolphins from India (Kannan et al., 1997b) and dolphins from Mediterranean Sea (Focardi et al., 2000). In other marine mammals, low concentrations were detected in seals from USA (Kajiwara et al., 2001) and Norway (Berge et al., 2004). However, significant concentrations accumulated in sea otters found along the coasts of USA and Russia (Murata et al., 2008).

Accumulation patterns of organotins by aquatic organisms vary according to their life styles. Stäb et al. (1996) showed that phenyltin concentrations were higher in benthic species while butyltin concentrations were higher in pelagic ones. So, they indicated that TBT is passed on primarily via the water, while TPT is passed on to a larger extent via the sediment. Takahashi et al. (2000) found that concentrations of butyltins were higher in migratory fish species moving up to the upper 200 m at night for feeding than non-migratory species living in deeper water. Shallow-water organisms have also been found to contain higher butyltin compounds than deep-sea ones from Suruga Bay, Japan (Takahashi et al., 1997) and from the northwestern Mediterranean Sea (Morcillo et al., 1997; Solé et al., 1998; Borghi and Porte, 2002). Avian TBT concentrations, generally measured in liver or kidney, were higher in birds from coastal sites than in those of oceanic habits. Nonetheless, Laysan albatross from the North Pacific Ocean had TBT concentrations as high as 43 ng/g w.w. in their liver (Guruge et al., 1997). Table 2 summarizes selected recent data for different biota and locations.

2.2.3.3. Biomagnification

Algae may take up TBT from seawater, followed by its stepwise transfer to primary consumers and upper predators, leading to its accumulation within the food web. ΣBT concentrations in sea water, sediment, plankton, primary consumers (benthic molluscs and crustaceans), small fish (fish feeding mainly zooplankton), large fish (fish showing a carnivorous habit), birds and marine mammals from three ecosystem are shown in Figure 2. A comparison of BTs in seawater and various organisms indicates that high concentrations are found in lower trophic levels (Takahashi et al., 1999). Zooplankton is an important trophic link, acting as a primary consumer and allowing the energy and carbon transfer along the food chain. Bioconcentration factors (BCF) are one way to compare bioaccumulation among different organisms. Concentration ratios of BT residues in organisms relative to the surrouding seawater (BCFs) are up to 35×10^4 for algae (Huang et al., 1993), 4×10^4 for clams (Gomez-Ariza et al., 1999), 4×10^4 for crabs (Kannan et al., 1995) and 5×10^4 for fish (Takahashi et al., 1999). In higher trophic animals such as large fish and marine mammals, relatively low BT bioconcentration was observed (Strand and Jacobsen, 2005). Biomagnification of organotins through food chains has been found in higher trophic aquatic organisms, such as myctophid fish (Takahashi et al., 2000), marine mammals and fish-eating birds (Kannan et al., 1996; Stäb et al., 1996; Kannan and Falandysz, 1997; Strand and Jacobsen, 2005).

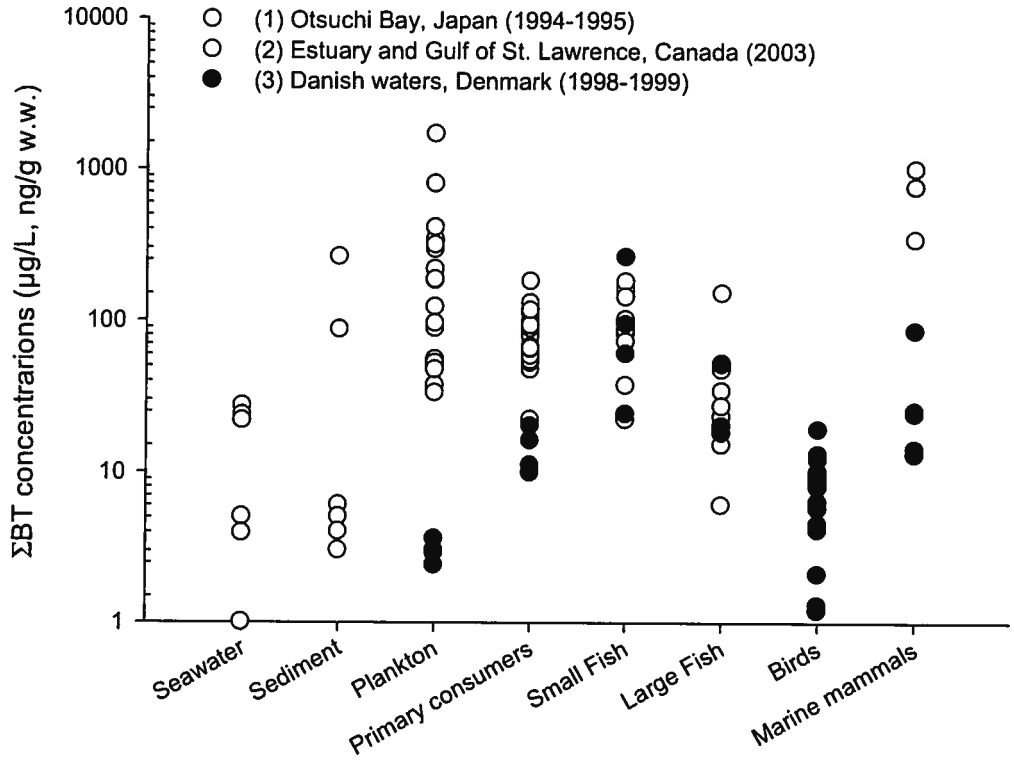


Figure 2. Accumulation profile of butyltin residues in three ecosystems. Data are from the following sources: (1) (Takahashi et al., 1999); (2) (Michaud and Pelletier, 2006); (3) (Strand and Jacobsen, 2005).

2.2.3.4. Degradation in Tissues

The ability of animals to degrade organotins to a variety of metabolites appears to be a widespread phenomenon, although its efficiency varies among species (Lee, 1985). For example, the polar seastar, can metabolize TBT by dealkylation to di-, mono-butyltin and finally to inorganic tin (Sn^{4+}) (Mercier et al., 1994). Digestive cell lysosomes are involved in the process of TBT excretion as butyltins which are mainly accumulated in pyloric caeca (Békri and Pelletier, 2004). Temperate sea anemones also possess the ability to metabolize TBT into DBT and further into MBT (Mercier et al., 1998). Although the role of butyltin-degrading microorganisms possibly present in the gut of the animals cannot be completely discarded, metabolism of TBT through a mixed-function oxidase system is equally conceivable. The presence and activity of such a system have often been recognized in marine invertebrates (den Besten et al., 1990; Lee, 1991) and were

recently observed in the scleractinian coral Favia fragum (Gassman and Kennedy, 1992), a close relative of sea anemones. In temperate sea anemones either butyltins were efficiently excreted or Sn was stored in unidentified chemical forms, as previously suggested for starfish (Mercier et al., 1994) and algae (Maguire et al., 1984; Lee et al., 1989). Mercier et al. (1996) demonstrated that the zooxanthellae present inside tissues of a symbiotic sea anemone are the principal mediators of butyltin regulation and degradation, during both food and water contamination. The expulsion of zooxanthellae was correlated with the depuration of butyltin (Mercier et al., 1997). TBT is mainly metabolized by the microsomal cytochrome P450 dependent monooxygenase (MO) system to hydroxylated compounds, leading to the formation of DBT and MBT and to their excretion as conjugated soluble metabolites (Bryan et al., 1993; Lee, 1996). The presence of cytochrome P450 and other MO system components in echinoderms was confirmed by den Besten et al. (den Besten et al., 1990). The activity of cytochrome P450 in pyloric caeca of asteroids was similar to that in other marine invertebrates (Lee, 1981; Livingstone and Farrar, 1985; Den Besten and Livingstone, 1989).

Mollusks in general, in contrast to crustaceans, show a very limited ability to metabolize TBT. Degradation by bivalves to DBT or MBT is commonly considered as low (Widdows and Page, 1993; Fent, 1996). For example, Laughlin et al. (1986) observed the slow and inefficient metabolism and elimination process of TBT by blue mussels. Oysters exposed to ^{14}C-TBT in water for up to six days resulted in only a small amount of the TBT being metabolized, primarily to dibutyltin (Lee, 1985). Oysters and other bivalves have very low mixed function oxygenase activity which correlates with the slow rate of toxicant oxidation in bivalves (Livingstone and Farrar, 1985; Lee, 1996).

Relatively few aquatic invertebrates have been studied to determine their capacity to metabolize TBT. A number of decapod crustaceans including blue crabs, spider crabs and shrimps can metabolize TBT which enters via the food or water (Lee, 1985, 1989; Rice et al., 1989). Stomach and hepatopancreas microsomes from crabs metabolize pollutants via their cytochrome P-450 system (James and Boyle, 1998). Crustaceans and fish are capable of enzymatic transformation of TBT into hydroxybutyldibutyltin first, then into dibutyltin and monobutyltin. Nevertheless, the possibility should not be neglected that exposure to high TBT concentrations in high contaminated areas may inhibit the detoxification mechanisms common to TBT.

A number of studies have shown that fish are able to metabolize TBT up taken via food or water (Lee, 1985). The fish liver is the primary organ for the metabolism for

TBT with subsequent transfer of conjugated metabolites to the bile (gall bladder) followed by the rapid elimination of these by-products. The speed of clearing is higher in liver than in muscle (Lee, 1991). In contaminated areas, liver may contain more BTs because of a constant input and transfer to other organs is a slower process (Harino et al., 2000). In a relatively low contaminated area, such as the St. Lawrence ecosystem (Michaud and Pelletier, 2006), liver can depurate more rapidly than muscle retaining MBT, the last step of TBT degradation. The main steps in this reaction involve the binding of TBT to oxidized cytochrome P-450 (Fe^{3+}) followed by the reduction of the complex to cytochrome P-450 (Fe^{2+}) which then interacts with oxygen. A hydroxylated substrate, e.g. beta-hydroxy-butyldibutyltin, and a molecule of water leave the now re-oxidized cytochrome P-450. The oxidized substrate P-450 complex is reduced by two electrons from NADPH carried by NADPH cytochrome P-450 reductase.

In studies of butyltins in birds, authors observed that moulting of feathers, which contain up to 30% of the TBT body burden, provides an important mechanism for detoxification (Guruge et al., 1997). The lower ratios of MBT:TBT and DBT:TBT observed in mammals relative to birds suggests that mammalian metabolism and excretion of TBT is less efficient than in birds (Iwata et al., 1997). The differences in accumulation patterns between species at the same trophic level, which are feeding on almost the same diet, could be related to species-specific capabilities to metabolise and eliminate organic contaminants such as organotins. This includes species-specific differences in the activity of the cytochrome P450-mediated mixed function oxygenase, which is suggested to be the primary enzyme system responsible for the metabolism of organotins and other organic contaminants (Lee, 1991; Fent, 1996; Tanabe, 2002). These differences are also reflected in the composition of butyltin since TBT contributes significantly to the butyltin burden in the liver of harbour porpoise but not in the liver of fish-eating birds and harbour seal, where the break-down products, DBT and MBT, are generally dominant (Strand and Jacobsen, 2005). That birds and seals have a high metabolic capacity for organic contaminants in comparison to cetaceans has also been suggested in other studies (Tanabe, 2002). An additional reason for the lower accumulation potential in birds and seals compared to cetaceans is the possibility of eliminating significant amounts of organotin by moulting of feathers or hair (Kim et al., 1996; Guruge et al., 1997). However, beluga whales from the St. Lawrence Estuary accumulated relativey low concentrations from their environment (St.-Louis et al., 2000).

2.3. Toxicity to marine organisms

Given their role in preventing the growth of sessile invertebrates on boat and ships, the toxic potentials of organotins to marine organisms are well documented (Fent, 1996). Test organisms have included fish, shrimps, crabs, molluscs, and tunicates. As a general rule, alkyltin toxicity decreases along with the number of alkyl radicals: tri-Sn > di-Sn > mono-Sn > SnO_2. Notable exceptions to this rule were reported by several authors who showed that some organisms were more sensitive to DBT than to TBT (Cima et al., 1995; Bouchard et al., 1999; Frouin et al., 2008). Trisubstituted organotins are extremely toxic to aquatic life for a large variety of organisms as shown *in vivo* and *in vitro* (Fent, 1996).

2.3.1. Algae and seagrass

Toxic effects of organotin compounds on microalgae have been reported by many investigators (Wong et al., 1982; Walsh et al., 1985; Beaumont and Newman, 1986; Huang et al., 1993; Marsot et al., 1995). TBT suppressed growth of the centric diatoms *Skeletonema costatum* and *Thalassiosira pseudonana*, the EC_{50} concentrations at low cell density being about 0.35 and 1.15 µg/L, respectively (Walsh et al., 1985). By comparison, the EC_{50} concentrations for dibutyltin dichloride (DBTC), and tetrabutyltin (TeBT) in *S. costatum* were 40 and 17 µg/L, respectively. However, this observation is surprising since *S. costatum* has the ability to degrade TBT to DBT and other products (Lee et al., 1989). Algae showed acute and chronic effects at concentrations of a few 100 ng/L or less (Beaumont and Newman, 1986). On the contrary, growth in microalgae *Dunaliella tertiolecta* and *Pavlova lutheri* cultures was only slightly affected at 0.1 µg/L and was not totally prevented at 1 µg/L. *D. tertiolecta* appears to have a very limited ability to degrade TBT (Lee et al., 1989). After an exposure to 0.12 µg/L TBTC, a marine phytoplankton community from a northern environment was affected in their physiological functions (i.e., photosynthetic efficiency and cell division) with a subsequent decrease in apparent growth rate and increase in cell size (Sargian et al., 2005). The effects of TBT from contaminated sediment on seagrass *Ruppia maritima* showed a strong 60% reduction of net photosynthetic activity (Jensen et al., 2004).

2.3.2. Invertebrates

Previous research has shown that *in vitro* exposures of the colonial ascidian, Botryllus *schlosseri*, to TBT and DBT (1 μM) inhibited phagocytosis; furthermore, exposure to MBT (10 μM) resulted in a slight but significant decrease of this activity (Cima et al., 1995). DBT was significantly more effective than TBT. Immunotoxicity of organotins, reflected by altered phagocytic activity, chemotaxis or reactive oxygen species generation, have been identified in tunicates, *Styela plicata* (Raftos and Hutchinson, 1997), *Ciona intestinalis* (Cooper et al., 1995) and *Botryllus schlosseri* (Cima et al., 1997). The embryonic and larval stages of marine invertebrates are less tolerant to toxicants than adults, and have been used for assessing the biological quality of marine water and sediments. In tunicates, organotin complexes (MBT, DBT and TBT) have been shown to affect development and differentiation (Mansueto et al., 1993a; Mansueto et al., 1993b). Cima et al. (1996) have shown that organotins significantly affect all stages of ascidian development in a dose- and time-dependent manner and that the most sensitive stages were gastrula and neurula. These compounds were able to block development, giving rise to anomalous embryos with irreversible effects. The order of inhibition appears to be strongly dependent on the organotin liposolubility: TBT>DBT>MBT and TPT>TCT. However, the concentrations used (0.1-10 μM) were higher than those having effects in mammals and fish, probably because in tunicate embryos, the egg envelopes act as protective barrier against contaminants in sea water (Mansueto et al., 1984).

There is some published information on the effects of organotin compounds on marine invertebrates, including a few echinoderms (Depledge and Billinghurst, 1999). A study by Békri and Pelletier (2004) demonstrated that in polar seastar the exposure to BTs decreased significantly the immune performance, phagocytic activity as a toxic endpoint. A previous study described reproductive impairment in Leptasterias polaris fed TBT-contaminated mussels for 53 days (Mercier et al., 1994). This article revealed smaller mature oocytes and thinner epithelium in the gonads of the contaminated individuals compared to control organisms. A study of the influence of TBT and TPT on arm regeneration in the brittle star, *Ophioderma brevispina*, revealed some evidence of inhibition at 10 ng/L and significant inhibition at 100 ng/L (Walsh et al., 1986). TPT affects skeletal development during arm regeneration of the echinoderm *Antedon mediterranea* (Barbaglio et al., 2006). This observation is in agreement with a previous study, which showed that it could affect significantly skeletogenesis in the larvae of the sea urchin *Paracentrotus lividus* (Moschino and Marin, 2002). Toxicity tests on the sea

urchin *P. lividus* emphasized that TPT and TBT can cause critical damage to sperm motility (Moschino and Marin, 2002; Novelli et al., 2002).

Sea anemone, *Aiptasia pallida*, exposed to TBT (50 ng/L via seawater or 25 ng/g via food) for 28 days, showed a decreasing concentration of zooxanthellae within their tentacles, along with an increased number of bacterial aggregates in the ectoderm (Mercier et al., 1997). Moreover, a significant decrease in the mitotic activity of zooxanthellae was observed, which may alternatively reflect the harmful effect of butyltin on the health of the zooxanthellae, since TBT is known to disturb the growth of algae (Beaumont and Newman, 1986). The loss of zooxanthellae after exposure to low concentrations suggests that the algae-cnidarian symbiosis is quite sensitive to TBT and to its derivative, DBT. In corals, visual bleaching of branchlets of *Acropora formosa* was observed after an exposure to sediments containing 160 mg/kg (366 ng/L in seawater), but an overall reduction in the symbiotic zooxanthellae population was not observed in surviving corals (Smith et al., 2003).

Bushong et al. (1990) had estimated in the marine copepod *Acartia tonsa* a no observed effects concentration (NOEC) for TBT of 12 ng/L. However, Kusk and Petersen (1997) found that TBT inhibited the developmental rate of larvae of A. tonsa at 3 ng/L.

Lau et al. (2007) examined the lethal effect of TBT on five developmental stages of the marine polychaete, Hydroides elegans. The 48-h LC50 for the eggs, 2-cells, trochophores, juveniles and adults were 0.15-0.2, 0.77-1.23, 2.11-2.65, 2.63-3.12, 4.04-4.71 μg TBT/L, respectively. Mattozo et al. (2002) showed that lysosomal activity was significant reduced at 0.05 μM TBT in the marine worm *Sipunclus nudus* and that the percentage of coelomocytes with ingested neutral red dye was also inhibited at 1 μM.

Some marine benthic invertebrates are also very sensitive to TBT in sediments. Matthiessen and Thain (1989) found that populations of the polychaete, *Scolopos armiger*, and the amphipod, *Urothoe poseidonis*, were reduced in sediment contaminated with 1 mg TBT/kg. Evidence obtained to date suggests that copepods are the most sensitive among crustaceans. The 48-h LC_{50} values for survival, was 1.2 to 6.6 μg TBT/L for the caprellids and 17.8 to 23.1 μg TBT/L for the gammarids, indicating caprellids are more sensitive to TBT than gammarids. However, no sex-specific differences of the acute toxicity of TBT were observed in the caprellids (Ohji et al., 2007). Both the male and female ratios changed dramatically when the caprellid amphipod, *Caprella danilevkii*, was exposed to 10 ng TBT/L during the embryonic stage (Ohji et al., 2002). Reproductive inhibitions such as loss of brood and inhibition of oogenesis

occurred even at exposures of 10 to 100 ng TBT/L in both parental females and their female offsprings (Ohji et al., 2002). Lignot et al. (1998) observed the acute toxicity of TBTO in shrimp, *Penaeus japonicus*, at several developmental stages. The 24 and 48-h LC_{50}s values ranged from 2.03 µg/L (1.7-2.4) and 0.88 µg/L (0.8-1.0) for nauplii (larvae) to 773 µg/L (344-1823) and 708 µg/L (361-1608) for juveniles. Exposure to TBT induced in blue crab, *Callinectes sapidus*, the expression of a CYP 3A-like protein in hepatopancreas (Oberdörster et al., 1998) and altered the expression of P450 isozymes and the pattern of testosterone hydroxylation.

Mollusks are known to be the most sensitive species to organotin exposure. Effects on larval development of bivalves as well as disturbances in gastropods sexuality were observed at ng/L concentrations in seawater. The first adverse effects of TBT on mollusks were observed, in the mid 70s, in Crassostrea gigas at the Bay of Arcachon (France), one of the centers of oyster aquaculture in Europe with ball-shaped shell deformations in adults and a drastic decline of annual spatfall (Alzieu et al., 1980). Observations in the natural environment revealed that the larvae survived only a few days, whereas larvae from the Bay waters transferred to the laboratory in seawater collected outside the bay were developing normally. Shell deformities in oysters, but also in other bivalve groups, have been used as a biological marker of TBT effects in the following years (Oehlmann and Schulte-Oehlmann, 2003; Medakovic et al., 2006). Another TBT effect in mollusks was initially described in a number of regions worldwide in the early 1970s without identifying the organotin compound as the causative agent at that time: a virilisation of female prosobranchs, which has been termed as imposex (Smith, 1971), with this condition leading to reproductive failure and consequently population decline (Bettin et al., 1996; Gibbs and Bryan, 1996; Matthiessen and Gibbs, 1998). Imposex is characterized by the formation of a penis and/or vas deferens in females of gonochoristic prosobranch species and is induced at lower concentrations than all other described TBT effects. Actually, imposex is known to occur in more than 150 prosobranch species (Bettin et al., 1996). The embryonic and larval stages of marine invertebrates are less tolerant to toxicants than adults. In young and sexual immature specimens of some muricid species a protogyne sex-change can be induced by TBT concentrations higher than 10 ng/L in *Nucella lapillus* (Gibbs et al., 1987) and above 2 ng/L in *Ocinebrina aciculata* (Oehlmann et al., 1996). The periwinkle *Littorina littorea* develops a closely related virilisation phenomenon as a response to TBT exposure, termed as intersex (Bauer et al., 1995). Intersex females are either

characterized by male features on female pallial organs, specifically by an inhibition of the ontogenic closure of the pallial oviduct; alternately female sex organs are supplanted by the corresponding male formations. In the marine rock shell *Thais clavigera* (Horiguchi et al., 1997) not only TBT but also TPT can promote imposex, while in other prosobranch species, such as *N. lapillus* and *Nassarius reticulatus*, TPT did not induce this virilisation phenomenon (Schulte-Oehlmann et al., 2000). Immunotoxic effects of organotins were found in mussels. Hemocytes of mussels and other bivalves are primarily responsible for defence against parasites and pathogens. St Jean et al. (2002) used haemocyte functions to determine the effect of low concentrations (1, 3 and 6 ng/L) of TBT and DBT on the immune system of adult *M. edulis* over 11 days. Their results support the hypothesis that both TBT and DBT can cause modulations of the internal defence system of mussels at concentrations as low as 1 ng/L after only a few days of continuous exposure. The negative effects of organotins on phagocytosis at low dose (0.05 μM) were observed in the clam *Tapes philippinarum* (Cima et al., 1998). In addition to effects on the internal defence system Jha et al. (2000) found genotoxic effects of TBT to embryo-larval stages of *M. edulis*. DNA damage in haemocytes of the mussel *M. edulis* (Hagger et al., 2005), in erythrocytes of the mollusc, *Scapharca inaequivalvis* (Gabbianelli et al., 2006) and DNA fragmentation in gill cells for the mussel *Mytilus galloprovincialis* (Micic et al., 2002) were reported after exposure to TBT.

2.3.3. Fish

Inhibition of cytochrome P450 by TBT and TPT has been demonstrated in fish both *in vivo* (Fent and Stegeman, 1993) and *in vitro* (Fent and Stegeman, 1991; Fent et al., 1998). The conjugation of testosterone was also significantly inhibited in fish (Morcillo et al., 2004). TBT-induced masculinisation has been seen in genetically female Japanese flounder (*Paralichthys olivaceus*) with concomitant suppression of the aromatase gene expression (Shimasaki et al., 2003). Zebrafish (*Danio retio*) early life stages are extremely sensitive to TBT. Nominal levels of 0.1 ng/L and higher can cause a high incidence of abnormal sperm and significantly bias the sex ratio towards males (McAllister and Kime, 2003). Moreover, TBT-induced dose dependent sperm abnormalities were seen. Fertilization success was reduced and sexual behaviour altered in male Medaka, exposed to TBT (Nakayama et al., 2004). The immunotoxicity of organotin compounds is only partially understood in fish. Rice et al. (1995) report an acute exposure to TBT having an effect on immunotoxicity of channel catfish (Ictalurus

punctatus). Humoral immunity and non-cytotoxic cells functions were affected at 1 mg TBT/kg (Rice et al., 1995). Fish are more susceptible in their early developmental stages (Fent, 1992; Fent and Meier, 1992). TBT is considered to be the most toxic organotin compound for most species, and its toxicity decreases with progressive dealkylation to di- and mono-butyltin. However, O'Halloran et al. (1998) hypothesized that DBT is a more potent immunotoxin than TBT in fish as demonstrated *in vitro*.

2.3.4. Birds

Data on the toxicity of organotins in birds are scarce. Dietary exposure of Japanase quail (*Coturnix coturnix japonica*) to TBTO resulted in embryotoxicity, reduced hatching success, and chick deformities. In addition, a decrease in serum aspartate aminotransferase, in calcium, in follicle-stimulating hormone, and in hepatic microsomal7-ethoxyresorufin (EROD) activity were recorded (Coenen et al., 1992; Schlatterer et al., 1993), although these studies were conducted at exposure doses above 24 µg TBT/g diet which have little significance when compared to field exposures for wild birds. It is not known if disrupted metabolism of steroids occurs in birds following organotin exposure. More toxicological studies are needed to evaluate the potential effects of butyltins in birds. Gentoo penguins (*Pygoscelis papua*) treated with trimethyltin (TMT) (15 mg/kg body mass), showing increase in proteins and in serum enzymes, aspartate transaminase and alanine transaminase (Najle et al., 2006).

2.3.5. Marine mammals

The inhibition of hepatic cytochrome P450 and enzyme activities by low levels of TBT exposure was noted in an experiment in vitro using fresh liver samples of Dall's porpoises and Steller sea lions (Kim et al., 1998). Decreases in P450 content and in the activities of ethoxyresorufin O-deethylase (EROD, catalyzed by CYP1A subfamily) and of penthoxyresorufin O-depentylase (PROD, catalyzed by CYP2B subfamily) by TBT were observed. EROD activity was more sensitive to TBT than were P450 content and PROD activity, indicating a specific mode of action of TBT on different P450 forms. DBT and MBT had no inhibitory effect on EROD activity, indicating that the inhibition of enzyme activity in hepatic microsomes of marine mammals is mainly induced by TBT (Kim et al., 1998). However, TBT concentrations that affect P450 contents and activities were higher than the values found in the liver of various marine mammals. Organotins were

suspected to exert some immunotoxic effects in marine mammals (Kannan et al., 1997a). In harbour seals, concentrations of 100 nM of DBT and 200 nM of TBT inhibited lymphocyte proliferation (Frouin et al., 2008). These results were similar to those reported by Nakata et al. (2002) on the immunotoxic effects of butyltins on marine mammal (pinnipeds and cetaceans) lymphocytes. Con A-stimulated mitogenesis was significantly suppressed when cells were exposed to 330 nM of DBT and 300 nM of TBT (Nakata et al., 2002). Phagocytic activity of harbour seal immune cells was impaired at 100 nM of DBT but TBT had no effect on phagocytosis (Frouin et al., 2008). DBT and TBT exposure decreased Natural Killer (NK) cell activity at 50 and 100 nM, respectively (Frouin et al., 2008). These authors hypothesized that DBT is more immunotoxic than TBT in marine mammals.

2.4. Mechanisms of action of organotins

2.4.1. Algae

There have been few reports on the destruction of submicrostructures of algae by organotins; however, this point is crucial for the elucidation of toxicity mechanisms. Huang et al. (1996) assessed submicrostructures of a green algae, *Platymonas sp.*, following tributyltin chloride (TBTC) exposure (0.2 µg/L). They reported an inhibition of the phosphorylation in photosynthesis and a deformation of the mitochondrion suggesting that TBTC affected the membrane of the mitochondrion, causing membrane swelling and increasing penetrability. Huang et al. (1997) proposed a toxicity mechanism for a series of organotins against two green algae based on QSAR (Quantitative-structure activity/property relationship) equations. The authors reported that the toxicity of the organotins was mainly controlled by the lipophilicity of the compounds with the electronic property playing a minor role. TBT affects the nitrogen metabolism in algae which is directly proportional to algal productivity. Zihihui and Guolan (2000) related TPT toxicity to nitrate reductase activity. TPT caused the algal cells to break, and the cellular inclusions including nitrate reductase were released into the culture solution, which resulted in that the activity of nitrate reductase out side was higher than that inside the algal cells.

Table 2. Global occurrence of organotins in biological tissues (ng/g (dry weight) unless indicated otherwise)

Biological sample	Tissue	Location (year)	Levels of organotins compounds				References
			MBT	DBT	TBT	TPT	
Plankton							
Bladder wrak (*Fucus vesiculosus*)		North/Baltic Sea (1985-1999)		<3-4	2-6	<5-14	Rudel et al. 2003[a]
Zooplankton (different species)		Estuary/Gulf Canada (2003)	<0.05-312	32-453	<0.05-68		Michaud and Pelletier 2006
Plankton (different species)		Otsuchi Bay, Japan (1994-1995)	23-200	6.9-300	23-1200		Takahashi et al.1999[a]
Macroalgae (different species)		Estuary, Canada (2002)	<d.l.-6.17	<d.l.-1.92	<d.l.-6.52		Mamelona and Pelletier 2003
Invertebrates							
Amphipod (*Caprella* spp.)		Coast, Japan (1997-1999)	15.7±22.6	8.0±9.0	32.0±48.0		Takeuchi et al. 2004[a]
Blue mussel (*Mytilus edulis*)	body	North/Baltic Sea (1985-1999)	<3-8	<3-7	9-21	<5-98	Rudel et al. 2003[a]
	body	Baltic Sea (1998)	<d.l-4.7	05.-24	2.2-38.9		Albalat et al. 2002[a]
Mussel (*Mytilus galloprovincialis*)	body	Coast, Portugal (1999-2000)	<7.9-41	<2.5-18	<5.7-489		Díez et al. 2005
	body	Port, Italy (1997-1998)	540-2190	690-3720	730-5790		Rivaro et al. 1999
	body	West coast, Portugal (2000)	<d.l.-605	<d.l.-345	11-789	<d.l.-16	Barroso et al. 2004
	body	North coast, Japan (2003)	0.83-2.9	0.83-3.1	0.77-11	0.08-0.62	Ohji et al. 2007[a]
Mussel (-)	body	North West coast, Spain (-)	8.7-13.2	20.2-25.7	52.8-96.1		Üveges et al. 2007
Clam (*Meretrix meretrix*)	body	Harbour, Vietnam (2003)	2.8-18.4	4.4-26.6	3.8-15.1		Nhan et al. 2005[a]
Clam (*Meretrix* spp.)	(n.a.)	Coast, Vietnam (2002)	0.1-44	0.5-10	1.4-56	<0.1-3.1	Midorikawa et al. 2004[a]
Oyster (*Crassostrea gigas*)	body	Aquaculture area, Taiwan (2002)	<d.l.-406.6	<d.l.-280.9	<d.l.-417.2		Hsia and Liu 2003[a]
Oyster (-)	body	North West coast, Spain (-)	2.9-582.4	7.6-440.7	98.4-192.8		Üveges et al. 2007
Bivalves (*M. edulis/C. gigas*)	body	Coast, Korea (1997-1998)	<d.l.-461	23-699	16-1610	20-1011	Shim et al. 2005
Gastropod (*Hexaplex trunculus*)	body	Lagoon Venice, Italy (2002)	24-74	34-151	44-117	2.4-29	Pellizzato et al. 2004
Starfish (*Asteria pectinifera*)	body	Coast, Korea (1997-1998)	51-2860	8-139	<d.l.-323	8-834	Shim et al. 2005
Starfish (*Asteria amurensis*)	body	Coast, Korea (1997-1998)	103-1520	45-751	14-797	23-1560	Shim et al. 2005
Ascidian (*Halocynthia roretzi*)	body	Bay, Japan (1994-1995)	<9	13	42		Takahashi et al. 1999[a]
Sea urchin (different species)	body	Bay, Japan (1994-1995)	<9	5.4-11	17-41		Takahashi et al. 1999
Green urchin (*S. droebachiensis*)	Gut	Estuary, Canada (2002)	10.46-174.48	0.8-35.39	7.44-123.94		Mamelona and Pelletier 2003
Squid (different species)	body	Deep-sea, Atlantic (2003)			<1-26		Unger et al. 2008

Fish							
Eelpout (*Zoarces viviparus*)	muscle	North/Baltic Sea (1985-1999)			10-22	5-60	Rudel et al. 2003[a]
Flounder (*Platichtys flesus*)	liver	Baltic Sea (1998)	<d.l.-18.5	4.7-286.1	6.3-34.9	2.8-29.7	Albalat et al. 2002[a]
Fish (several species)	muscle	Coast, Malaysia (1998)	2.3-7.4	<1,3-1.3	2.4-190		Sudaryanto et al. 2004[a]
Fish (several species)	muscle	Port Osaka, Japan (1996)	25-83	2-18	20-182	<1-130	Harino et al. 2000[a]
Fish (several species)	liver	Mediterannean, Spain (1996)	<1-54.5	4-67	1-52.1	<1.4-1430	Borghi and Porte 2002[a]
Fish (several species)	muscle	Bay, Japan (1994-1995)	<9-25	2.1-13	4.2-13		Takahashi et al. 1999[a]
Skipjack tuna (*K. pelamis*)	liver	Asia (1996-2001)	<1.8-38	<2.4-170	3.8-220		Ueno et al. 2004[a]
Bluefin tuna (*T. t. thynnus*)	liver	Coast, Italian (1992-1993)	21-53	9-440	18-150		Kannan et al. 1996[a]
Blue shark (*Prionace glauca*)	liver	Coast, Italian (1992-1993)	4-13	4.3-6	11-23		Kannan et al. 1996[a]
Birds							
Cormorant (*Phalacrocorax carbo*)	kidney	Lake Biwa, Japan (1993)	23-180	77-390	6-23		Guruge et al. 1996[a]
	liver	Lake Biwa, Japan (1993)	12-700	92-300	5-22		Guruge et al. 1996[a]
Seabirds (different species)	liver	Lake Biwa, Japan (1985-1994)	<5-700	<3-300	<1-40		Guruge et al. 1997[a]
Seabirds (different species)	kidney	Lake Biwa, Japan (1985-1994)	<5-180	<3-390	<1-34		Guruge et al. 1997[a]
Glaucous gull (*Laus hyperboreus*)	liver	Coast, Norwegian (1998)	<2-9.7	<3-26	<3	<2	Berge et al. 2004[a]
Marine mammals							
Killer whale (*Orcinus orca*)	liver	Hokkaido, Japan (2005)	16-152	16-556	6-25	<1-14	Harino et al. 2008[a]
Whales (different species)	liver	Coast, Thailand (1997-2002)	31-916	47-2870	37-1071	16-592	Harino et al. 2007
Harbour porpoise (*P. phocoena*)	liver	Denmark/Greenland (1998-1999)	<1-102	2-3689	<0.5-871	<3-62	Strand et al. 2005[a]
	liver	Coast, Norwegian (1988 & 1999)	4.2-59	9-469	18-156	<1-59	Berge et al. 2004[a]
Dall's porpoise (*P. dalli*)	liver	Bay, Japan (1994-1995)	50-120	180-600	110-310		Takahashi et al. 1999[a]
Bottlenose dolphin (*T. truncatus*)	liver	Coast, Italian (1992-1993)	150-200	800-1600	250-400		Kannan et al. 1996[a]
Dolphins (different species)	liver	Coast, Thailand (1997-2003)	16-98	30-588	12-471	<1-7	Harino et al. 2008
Dolphins (different species)	liver	Mediterranean (1992-1994)	3-205	<d.l.-434	<d.l.-4014		Focardi et al. 2000[a]
Cetaceans (different species)	liver	Coast, UK (1992-1998)	<3-35	19-230	<3-82		Law et al. 1999[a]
Beluga whale (*D. leucas*)	liver	Chukchi Sea (1989)	2.2-4.8	13.3-17.2	1.02-1.34		Point et al. 2007[a]
	liver	Estuary, Canada (1995-1998)	<d.l.-399	<d.l.-1613	<d.l.-467		St.-Louis et al. 2000
Caspian seal (*Phoca caspica*)	liver	Caspian Sea (2000)	<4.6-9.3	<2-11	0.49-6.8		Kajiwara et al. 2002[a]
Harbour seal (*Phoca vitulina*)	liver	Coast, Norway (1998)	2.3-27.3	2.9-22.8	<1-2.9	2.1-4.6	Berge et al. 2004[a]

	liver	Coast, USA (1991-1997)	<2.3-77	<2.4-27	0.69-2.2		Kajiwara et al. 2001[a]
Ringed seal (*Phoca hispida*)	liver	Coast, Norway (2000)	<1-2.2	<1-4.4	<1	<1	Berge et al. 2004[a]
Elephant seal (*M. augustrirostris*)	liver	Coast, USA (1991-1992)	9.5-19	50-78	0.55-2.1		Kajiwara et al. 2001[a]
California sea lion (*Z. californianus*)	liver	Coast, USA (1991-1997)	9.2-46	7.3-40	0.56-2.8		Kajiwara et al. 2001[a]
Dugong (*Dugong dugong*)	liver	Coast, Thailand (1998-2003)	15-381	4-2485	<1-20	1-13	Harino et al. 2007[a]
Sea otter (*Enhydra lutris*)	liver	Coast, USA/Russia (1992-2002)	1.9-610	2.4-2400	<0.33-1300	<0.1-0.89	Murata et al. 2008[a]
Polar bear (*Ursus maritimus*)	liver	Beaufort Sea (1996)	0.44-1.09	0.21-0.43	0.09-0.28		Point et al. 2007[a]

MBT: monobutyltin; DBT : dibutyltin; TBT : tributyltin; TPT : triphenyltin; <d.l. : below detection limit; n.a. : no data available

[a] wet weight

Table 3. Toxicity of organotins to selected sensitive aquatic biota

	Organism	Compound	Toxicity	Concentration	References
Acute toxicity					
	Alga (*Skeletonema costatum*)	TBTC	EC_{50}, 72 h	0.36 µg/L	Walsh et al. 1985
	Alga (*Skeletonema costatum*)	DBTC	EC_{50}, 72 h	40 µg/L	Walsh et al. 1985
	Alga (*Thalassiosira pseudonana*)	DBTC	EC_{50}, 72 h	181 µg/L	Walsh et al. 1985
	Alga (*Skeletonema costatum*)	TeBT	EC_{50}, 72 h	17 µg/L	Walsh et al. 1985
	Alga (*Skeletonema costatum*)	TPTC	EC_{50}, 72 h	0.92 µg/L	Walsh et al. 1985
	Alga (*Thalassiosira pseudonana*)	TPTC	EC_{50}, 72 h	1.34 µg/L	Walsh et al. 1985
	Alga (*Platymonas* sp.)	TBTC	EC_{50}, 96 h	0.31 ng/L	Huang et al. 1996
	Alga (*Platymonas* sp.)	DBTC	EC_{50}, 96 h	30.04 ng/L	Huang et al. 1996
	Alga (*Platymonas* sp.)	MBTC	EC_{50}, 96 h	38.35 ng/L	Huang et al. 1996
	Alga (*Platymonas* sp.)	TPTC	EC_{50}, 96 h	0.63 ng/L	Huang et al. 1996
	Microalga (*Nannochloropsis oculata*)	TBTC	EC_{50}, 24 h	0.258 µg/ L	Sidharthan et al. 2002
	Copepod (*Acartia tonsa*)	TBTO	LC_{50}, 96 h	1 µg/ L	U'Ren 1983
	Copepod (*Acartia tonsa*)	TBT	LC_{50}, 48 h	0.24 µg/L	Kusk and Pertersen 1997
Larva	Tunicate (*Ciona intestinalis*)	TBT	EC_{50}, 48 h	6.01-8.51 µg/L	Bellas et al. 2005
Larva	Sea urchin (*Parancentrotus lividus*)	TBT	EC_{50}, 48 h	0.273-0.363 µg/L	Bellas et al. 2005
Adult	Polychaete (*Hydroides elegans*)	TBT	LC_{50}, 48 h	4.04-4.71 µg/L	Lau et al. 2007
Adult	Caprellids (different species)	TBTC	LC_{50}, 48 h	1.2-6.6 µg/L	Ohji et al. 2002b
Adult	Gammarids (different species)	TBTC	LC_{50}, 48 h	17.8-23.1 µg/L	Ohji et al. 2002b
Larva	Shrimp (*Peanus japonicus*)	TBTO	LC_{50}, 48 h	0.8-1.0 µg/L	Lignot et al. 1998
	Brine shrimp (*Artemia salina*)	TBT	LC_{50}, 24 h	41.41 ng/L	Panagoula et al. 2002
Larva	Prawn (*Palaemon serratus*)	TBT	LC_{50}, 48 h	17.52 µg/L	Bellas et al. 2005
	Red Killifish (*Orizias latipes*)	TBTC	LC_{50}, 48 h	111 µM	Nagase et al. 1991
	Red Killifish (*Orizias latipes*)	TPTC	LC_{50}, 48 h	167 µM	Nagase et al. 1991
	Red Killifish (*Orizias latipes*)	DBTC	LC_{50}, 48h	19.1 mM	Nagase et al. 1991
	Red Killifish (*Orizias latipes*)	MBTC	LC_{50}, 48h	135 mM	Nagase et al. 1991
	Flounder (*Platichthys flesus*)	TBT	LC_{50}, 14 d	17.9 µg/L	Grinwis et al. 1998

Chronic toxicity

	Alga (*Skeletonema costatum)*	TBT	growth	0.1 µg/L	Beaumont and Newman 1986
	Alga (*Dunaliella terticolecta*)	TBT	growth	0.1 µg/L	Beaumont and Newman 1986
	Alga (*Pavlova lutheri*)	TBT	growth	0.1 µg/L	Beaumont and Newman 1986
	Alga (*Pavlova lutheri*)	TPTC	growth, IC_{50}	10 µg/L	Marsot et al. 1995
	Alga (*Pavlova lutheri*)	TPTC	growth	53.83 µg/L	St.-Louis et al. 1994
	Alga (*Spirulina subsalsa*)	TPT	growth, 8d, IC_{50}	15.63 µg/L	Zhihui and Guolan 2000
	Ascidia (*Botryllus schlosseri*)	TBT, DBT	PA	1 µM	Cima et al. 1995
	Ascidia (*Botryllus schlosseri*)	TPTC	PA/OB	1 µM	Cima et al. 1997
Embryo	Ascidia (*Ciona intestinalis*)	TBT	cleavage	32 µg/L	Gianguzza et al. 1996
Embryo	Ascidia (*Styela plicata*)	TBT	cleavage	320 µg/L	Cima et al. 1996
Sperm	Sea urchin (*Paracentrotus lividus*)	TPT	fertilization	0.1 µg/L	Moschino and Marin 2002
Egg	Sea urchin (*Paracentrotus lividus*)	TPT	embryotoxicity	1.5 µg/L	Moschino and Marin 2002
Sperm cell	Sea urchin (*Paracentrotus lividus*)	TBT	spermiotoxicity	5.83 µg/L	Novelli et al. 2002
Sperm cell	Sea urchin (*Paracentrotus lividus*)	TPTA	spermiotoxicity	18.5 µg/L	Novelli et al. 2002
Embryo	Sea urchin (*Paracentrotus lividus*)	TBT	embryotoxicity	2.46 µg/L	Novelli et al. 2002
Embryo	Sea urchin (*Paracentrotus lividus*)	TPTA	embryotoxicity	1.17 µg/L	Novelli et al. 2002
Egg	Sea urchin (*Anthocidaris crassispina*)	TBT	development	0.1 µg/L	Kobayashi and Okamura 2002
	Crinoid (*Antedon mediterranea*)	TPT	skeletogenosis	0.225 µg/L	Barbaglio et al. 2006
	Sea anemone (*Aiptasia pallida*)	TBT	symbiosis	0.050 µg/L or 5 µg/g	Mercier et al. 1997
Larva	Copepod (*Acartia tonsa*)	TBT	development	0.003 µg/L	Kusk and Petersen 1997
Juvenile	Polychaete (*Hydroides elegans*)	TBT	growth	1 µg/L	Lau et al. 2007
Juvenile	Amphipod (*Caprella danilevskii*)	TBT	maturation	0.1 µg/L	Ohji et al. 2003
	Mussel (*Mytilus edulis*)	TBT	genotoxicity	0.5 µg/L	Hagger et al. 2005
	Fish hepatoma cells (PLHC-1)	TBTC	NR50; MTT_{50}	110 µM; 620 µM	Nagase et al. 1991
	Fish hepatoma cells (PLHC-1)	TPTC	NR50; MTT_{50}	170 µM; 400 µM	Nagase et al. 1991
	Fish hepatoma cells (PLHC-1)	DBTC	NR50; MTT_{50}	20 mM; 30 mM	Nagase et al. 1991
	Carp (*Cyprinus carpio*)	TPT	estradiol sulfation IC_{50}	17 µM	Thibaut and Porte 2004
	Carp (*Cyprinus carpio*)	TBT	estradiol sulfation IC_{50}	18 µM	Thibaut and Porte 2004

Adult	Marine mammals (different species)	TBT	Immunotoxicity	89 µg/L	Nakata et al. 2002
Adult	Harbour seal (*Phoca vitulina*)	TBT	Immunotoxicity	200 nM	Frouin et al. 2008
Adult	Marine mammals (different species)	DBT	Immunotoxicity	77 µg/L	Nakata et al. 2002
Adult	Harbour seal (*Phoca vitulina*)	DBT	Immunotoxicity	100 nM	Frouin et al. 2008

Note : EC_{50} : concentration required to induce a 50% effect; IC_{50}: concentration that induces 50% inhibition
PA: phagocytic activity; OB: oxidative burst

2.4.2. Invertebrates

For TBT, in tunicates, the mechanism of action follows the Ca^{2+}-dependent pathway through direct interaction of butyltin compounds with calmodulin (Cima and Ballarin, 2000), which causes inhibition of calmodulin-dependent Ca^{2+}-ATPase and consequently a substantial increase in cytosolic Ca2+ concentration (Cima et al., 2002). This irreversible process in turn triggers a cascade of events including 1) extensive oxidation of thiol groups (Cima and Ballarin, 2004), a process known to affect cytoskeletal proteins, which causes alterations in cell morphology (Cima and Ballarin, 2000); 2) inhibition of both phagocytosis and associated processes, such as the respiratory burst (Cima et al., 1995); and 3) induction of apoptosis (Cima and Ballarin, 1999). In *Botryllus schlosseri*, TPT appears to act on membrane Ca^{2+} permeability, decreasing Ca^{2+} pump activity and increasing the intracellular Ca^{2+} concentration (Cima et al., 1997). Tujula et al. (2001) demonstrated that the effects of TBT on sub-cellular events are likely to disrupt the exocytosis of prophenoloxidase containing granules, and in doing so, decrease the amount of prophenoloxidase released per hemocyte. Once released from hemocytes, prophenoloxidase is proteolytically converted to its active form, phenoloxidase, which is the central component of an enzyme cascade that has been identified in both crustacean and tunicates (Arriza et al., 1995; Hauton et al., 1997). Some components of the phenoloxidase cascade can opsonise pathogens for phagocytosis. Moreover, monoclonal antibodies against hemocyte surface antigens have been used in conjunction with histological analysis in *Styela plicata* to demonstrate that TBT affects the frequency and morphology of hemocytes, implying an increase of refractile vacuolated cells in the circulating hemolymph (Radford et al., 2000). Theses cells are known to accumulate vanadium or iron for normal physiological purposes, possibly related to tunic formation or defence (Wright, 1981). Increase of refractile vacuolated cells implying that they may also be sites of TBT sequestration (Radford et al., 2000).

Exposure to low concentrations of TPT (0.05-1 µg/L) affects skeletogenesis in *A. mediterranea* by interfering with sex steroid activity and metabolism (Barbaglio et al., 2006). TPT (0.225 µg/L) was shown to produce a significant increase in testosterone level accompanied by a relevant decrease in the estrogen levels. In agreement with the known activity of TBT, TPT appears to interfere with steroid metabolism, possibly acting on synthesis by cytochrome P450-dependent aromatase or excretion rather than on

steroid receptors (Matthiessen and Gibbs, 1998). In echinoderms, TPT has an androgenic effect (Barbaglio et al., 2006). Both TBT and TPT strongly inhibited testosterone esterification in gonad microsomal fractions of *P. lividus* (Janer et al., 2005). In Lavado et al. (2006), TPT exposure led to a significant increase in fatty acid esterification of testosterone in *P. lividus* exposed to 225 ng/L. This study showed significant alterations of androgen metabolism in TPT-exposed sea urchins together with an inhibition of P450-aromatase activity, and significant changes in steroid levels in the coelomic fluid. The authors suggested a coordination of androgen metabolism and androgen synthesis in the sea urchin to maintain steroid levels in gonads stable after exposure to the androgenic compounds. Girard et al. (1997) found that TBT inhibits sea urchin egg cleavage by altering many of the cellular events related to cell division. The cytotoxicity of TBT often results in an arrest of cellular dynamics, leading to apoptosis or to a blocking of cell division (Girard et al., 1997) primarily occurring through an alteration of macromolecular syntheses or membrane-mediated processes controlling cell signalling. These processes consist primarily of a disruption of calcium homeostasis or calcium signalling (Girard et al., 1997; Girard et al., 2000). Girard et al. (1997) suggested that the TBT-induced decrease of the intracellular compartmentalisation of Ca^{2+} results mainly from an inhibition of the ATP-driven Ca^{2+} pump located at the membrane of the reticular compartment.

The effects of TBT exposure on *P. japonicus* osmoregulatory capacity proved to be time- and dose-dependent (Lignot et al., 1998). Rapid and significant decreases of the hyper- and hypo-osmoregulatory capacity (OC) appeared at both lethal and sublethal concentrations. Hypo-OC, however, was more negatively affected by TBT exposure than hyper-OC (Lignot et al., 1998). The effects of TBT on gill structures of the exposed shrimps might be one of the main factors responsible for the loss of the hypo- and hyper-OC. The gills and epipodites are probably, together with the digestive tract, the main tissues through which TBT enters the organism from the water and histological damages (e.g. to epithelial cells) can explain the imbalance in the plasma ion concentrations. Gill lamellae necrosis, nephrocyte hyperplasia and haemolymph congestion were observed (Lignot et al., 1998). Damage to epithelial cells (vacuolization), oedema and severe multiple epithelial necrosis were also noted to epipodites (Lignot et al., 1998). The severe injuries observed in gill lamellae and epipodites of *P. japonicus* can alter the surface permeability to water and/or to ions and therefore can be the factor responsible for the loss of the osmoregulatory capacity.

The cytochrome P450 monooxygenase system is a mutli-enzymatic system that plays a key role in the metabolism of both xenobiotic and endogenous compounds, such as hormones and fatty acids. It catalyses the hydroxylation of hundreds of structurally diverse compounds, whose only common feature appears to be the degree of lipophilicity. Several P450 isozymes are involved in the metabolism of xenobiotics, such as benzo[a]pyrene hydroxylation or 7-ethoxyresorufin-O-deethylation; whereas others are involved in the synthesis of sex hormones, including the aromatization step that converts androgens (testosterone/androstenedione) to estrogens (estrone/estradiol). In vertebrates, cholesterol is converted by a number of steps into androstenedione, which can then be converted either into testosterone or into estrone, the latter process being mediated by cytochrome P450-dependent aromatase (Hall, 1986). Furthermore, testosterone itself can be converted to 17β-estradiol, a step that is also catalyzed by aromatase, In other words, both estrogens and androgens are synthesized by essentially the same biochemical pathway and are structurally very similar. Blockage of any of these biosynthetic steps could lead to either elevated or depressed steroid hormone concentrations, which in turn could produce a cascade of undesirable effects. Although the hormonal systems of invertebrates are not as well understood as those of vertebrates, it is known that molluscs contain vertebrate-like steroids (Joose, 1984), and mollusc steroidogenosis is also similar to the vertebrate plan (De Longcamp et al., 1974). It has long been speculated that TBT causes imposex and intersex in gastropod molluscs by interfering with steroid biosynthesis rather than by mimicking the action of testosterone at the androgen receptor, but there are competing hypotheses about exactly how this may occur. The first possible mode of endocrine action of TBT in gastropods is the aromatase inhibition hypothesis which was consequently put forward by Spooner et al. (1991). These authors showed that although concentrations of progesterone and 17β-estradiol were not affected in female *N. lapillus* exposed for up to 42 days to 98 ng/L of TBT, testosterone concentrations became significantly elevated compared with controls after 28 days. Morcillo and Porte (1998) observed also testosterone levels in TBT-exposed clams were significantly higher than in control organisms. According to this hypothesis TBT acts as a competitive inhibitor of cytochrome P450-mediated aromatase, resulting in the increase of testosterone (Bettin et al., 1996) which would otherwide be metabolized (Matthiessen and Gibbs, 1998). The inhibition of the cytochrome P450 isozyme known as P450arom (CYP19A1, aromatase) can cause profound effects on the sexuality of vertebrates as demonstrated in Chinook

salmon (Oncorhynchus tshawytscha) by Piferrer et al. (1994). Because mollusc steroid biochemistry is similar to fish, inhibition of aromatose in gastropods could produce similar effects. Spectral changes of mussel cytochrome P450 after incubation with 1 mM TBT or 1 mM TPT were observed in Morcillo and Porte (Morcillo and Porte, 1997). However, the low levels of cytochrome P450 present in molluscs (14-45 pmol/mg protein) made the accurate determination of organotin-cytochrome P450 interaction difficult (Morcillo and Porte, 1997). In invertebrates, including molluscs, a multifunctional oxygenase (MFO) system exists which catalyses the conversion of androgens to estrogens (Kirchin et al., 1988; Livingstone et al., 1989; Livingstone et al., 1990). The same MFO system is believed to be responsible for the TBT debutylation (Lee, 1985). In gastropods, increases in testosterone levels were accompanied by an increase in penis length in TBT-exposed organisms (Spooner et al., 1991; Bettin et al., 1996). Moreover, a neuropeptide hormone termed Penis Morphogenic Factor (PMF) normally inducing male differentiation in molluscs has been postulated to induce imposex in female snails (Féral and LeGall, 1983). Past studies have shown that TBT is a neurotoxin that co-localizes in the ganglia which produces PMF (Bryan et al., 1989). This lends support to a hypothesis on a mechanism of imposex induction by TBT: release of PMF through TBT's neurotoxic action. TBT had strong effects on the development of imposex in the dog whelk in exposure experiment after hatching (Gibbs et al., 1988). Bettin et al. (1996) supposed that higher levels of TBT in the presence of natural levels of testosterone would competitively inhibit the cytochrome P450-dependent aromatase (rather than suppressing activity of the MFO system itself), thereby inhibiting the aromatization of the androgens androstenedione and testosterone to the estrogens estrone and 17β-estradiol. The former then builds up to levels that lead to imposex induction. This is consistent with the proposal that TBT acts as a competitive inhibitor of aromatase and does not suppress the MFO system itself.

In vitro and *in vivo* work by Ronis and Mason (1996) with the periwinkle *L. littorea* has led to the proposal of an alternative hypothesis to that of aromatase inhibition, i.e. the testosterone excretion-inhibition hypothesis. They demonstrated that cytochrome P450-dependent testosterone metabolism occurred but was only modestly affected by TBT. Even at 100 μM TBT, aromatase activity (as measured by estradiol formation) was inhibited by only 30 to 40% but irrespective of the presence of NADPH and there was an apparent TBT-dependent increase in androstenedione formation from testosterone. In the presence of NADPH, the main route of testosterone metabolism was via the 5α-

reductase pathway to its more active metabolite 5α-dihydrotestosterone, and this process was partly inhibited by 100 μM TBT. Morcillo and Porte (1998) studied the effect of TBT on Phase I metabolism of testosterone. In clam, R. decussata, TBT in vitro caused a significant decrease in the total metabolic rate of testosterone, as well as a change in the profile of the obtained metabolites. There was a decrease in the formation of androstenedione and an increase in the formation of hydroxylated metabolites (Morcillo and Porte, 1998). No significant effect was seen in the formation of estrone and estradiol due to aromatase activity (Morcillo and Porte, 1998). When the formation of estrone and estradiol from [^{14}C] testosterone was examined *in vivo*, an important decrease in the formation of both metabolites was evident (Morcillo and Porte 1998). These results indicate a strong decrease in the aromatization of testosterone to estrone and estradiol in TBT-exposed clams. Androstenedione, one of the male sex hormones, can be aromatized to estrogens in molluscs (Le Guellec et al., 1987) and was detected in addition to progesterone, testosterone, 17β-estradiol, estrone and estriol in *H. reticulata* (Bettin et al., 1996). The precise mechanism by which TBT is able to induce imposex or intersex has not been described unequivocally, although the weight of evidence at present suggests that competitive inhibition of cytochrome P450-dependent aromatase is probably occurring in TBT-exposed molluscs.

2.4.3. Fish

Because of their roles in the detoxification and activation of foreign compounds, alteration of the expressions of hepatic cytochrome P450 enzymes affects the potential risks and benefits of xenobiotics in general and of organotin compounds in particular, and is toxicologically important (Williams et al., 1998). It has been demonstrated that TBT may interact with *in vitro* and *in vivo* hepatic cytochrome P450 in marine fish, leading to inactivation of enzyme activity levels (Fent and Stegeman, 1993; Fent et al., 1998). Furthermore, recent investigations on the effect of TBT at lower concentrations than those reported to date from the subchronic test of fish have suggested an involvement of cytochrome P450 enzymes in TBT metabolism or the androgenic effects of TBT in fish (Mortensen and Arukwe, 2007). A further study on salmon hepatocytes (Mortensen and Arukwe, 2009) indicated that TBT produced significant decreases of vitellogen (Vtg) expression and modified the expression patterns of estrogen receptors. TBT produced differential expression patterns that were dependent on exposure time

and concentration for all salmonid AhR2 isoforms and also produced exposure- and time-specific modulations for isoforms CYP1A1, CYP3A, AhRR, and Arnt mRNA.

Several mechanisms could account for the loss and inactivation of CYP forms: 1) binding of organotins to CYP, resulting in direct destruction of the native enzyme, formation of P420 with subsequent breakdown, and inhibition of enzyme activity; and 2) inhibition of the NADPH cytochrome P450 reductase and NADH cytochrome P450 reductase affecting the electron transport for the oxidation of the substrate (Fent, 1996). A previous study in PLHC-1 fish hepatoma cells indicated that TBT inhibits CYP1A1 activity non-competitively which means that the inhibitor does not compete with the substrate for the binding site, but instead decreases the turnover rate (Brüschweiler et al., 1996). In another study, incubation of rainbow trout microsomes with 100μM TBT in vitro resulted in the formation of a type I difference binding spectrum (Fent and Butcheli, 1994). It was concluded that TBT and other organotin compounds may bind to amino acids such as cysteine, and probably histidine, resulting in perturbations at the substrate binding center and in inactivation of catalytic activity, or at additional sites of the enzymes leading to the loss of catalytic activity. The destruction of CYP1A integrity occurred in the absence of added NADPH, indicating that this was a direct effect of TBT and TPT and not the result of mechanism-based inactivation, or of the suicide processing of these substrates by CYP. Reader et al. (Reader et al., 1999) proposed that TBT could trigger apoptosis in rainbow trout hepatocytes through a step involving Ca^{2+} efflux from the endoplasmic reticulum or other intracellular pools and by mechanisms involving cysteine proteases, such as calpains, as well as the phosphorylation status of apoptotic proteins.

The neurotoxicity of TBT on the brain of marine fish *Sebastiscus marmoratus* was recently investigated (Zuo et al., 2009) and results showed that TBT can modulate amino acid neurotransmitters and N-methyl-D-aspartate receptor (NMDAR) signalling pathway. The alteration of expression of glutamatergic receptor NMDAR and components on its signalling pathway accompagnied with the change of total brain transmitter level indicated the importance of glutamatergic system in organotin toxicity. Other results showed that TBT exposure induced apoptosis in brain cells of three regions including the pallial areas of the telencephalon, the granular layer of the optic tectum, and the cerebellum (Zhang et al., 2008).

A specific tributyltin-binding protein type 1 (TBT-bp1) was discovered in the blood of the Japanese flounder, *Paralichthys olivaceus* and genomic studied indicated that

protein can be classified as alipocalin and is probably involved in extraction of TBT via mucus (Satone et al., 2008).

2.4.4. Marine mammals

Toxicity mechanisms of TBT have been extensively studied in terrestrial mammals (Cooke, 2006; Nakanishi, 2008) but few reports have described the critical target molecules for the toxicity and mechanisms of toxicity of organotin compounds in marine mammals. The data presented in Kim et al. (1998) demonstrate for the first time that in Dall's porpoises and Steller sea lions, TBT is an inhibitor of cytochrome P450 aromatase, the enzyme responsible for the conversion of androgens to estrogens. Since the cytochrome P450 aromatase is an evolutionarily highly conserved protein, the inhibition of its activity by TBT reported is presumably similar in all marine mammal species.

2.5. Conclusion

Restrictions for recreational boats resulted in a decrease of TBT levels in seawater of marinas, but it had a slow or marginal effect in coastal waters. However, in areas frequented by large ships, the organotin compounds continue to be input to the surface of sediment, such as in some harbors in Mediterranean and Asian countries and in Northern Europe. Organotins persisted and accumulated in sediment and biota. The worldwide contamination found also in coastal regions of developing countries, in the open oceans, in the deep seas and in the Arctic demonstrates the global occurrence of organotins. The high toxicity, persistence and bioaccumulation of TBT are of particular environmental concern. Androgenic activity and immunotoxicity of organotins in molluscs, fish and mammals were reported, indicating important effects on health of marine organisms. Due to the consumption of either contaminated drinking water and beverages, and in particular marine food a widespread human exposure to TBT and toxic effects on human health have been also documented (Antizar-Ladislao, 2008).

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3. IMMUNOLOGIE COMPARÉE

3.1. Phagocytose chez les nouveau-nés et adultes de phoques communs, gris et du Groenland

Article 1 : *Héloïse Frouin, Michel Lebeuf, Mike Hammill, Michel Fournier. Phagocytosis in pup and adult harbour, grey and harp seals. Veterinary Immunology and Immunopathology. (Accepté le 24 août 2009).*

Mon rôle a consisté à faire l'échantillonnage, les analyses, interpréter les données et écrire cet article à titre d'auteur principal. Article accepté pour publication dans.

RÉSUMÉ

L'immunologie des pinnipèdes est encore largement méconnue. Les effets de l'âge et le développement du système immunitaire chez les pinnipèdes nécessitent plus de recherches. La présente étude examinait l'activité et l'efficacité phagocytaire chez les leukocytes de phoques gris, commun et du groenland. Dans la première partie de l'étude, le sang périphérique était collecté sur des phoques commun (femelles d'âge divers) maintenues en captivité. Les résultats montrent une diminution de la phagocytose reliée à l'âge chez les femelles phoques communs dont l'âge varie de sub-adulte à adulte. Dans la seconde partie de l'étude, les changements dans la phagocytose ont été quantifiés au cours de la lactation chez les nouveau-nés phoques communs, gris et du Groenland et leurs mères (phoques gris et du Groenland). Chez les nouveau-nés du même âge, les leukocytes de phoques communs et du Groenland phagocytaient moins que ceux des nouveau-nés de phoques gris. L'activité et l'efficacité phagocytaire augmentait significativement du début au milieu de la lactation chez les nouveau-nés de phoques communs, et du début à la fin de la lactation chez les nouveau-nés de phoques gris, ce qui pourrait suggérer que le transfert de facteur(s) promouvant la phagocytose dans le colostrum est une caractéristique importante de la protection immunitaire temporaire pour les nouveau-nés. Au contraire, aucun changement dans l'activité et l'efficacité phagocytaire n'était observé chez les femelles lactantes des deux espèces examinées (phoques gris et du Groenland). À la fin de la lactation, aussi bien l'activité phagocytaire chez les nouveau-nés phoques gris et du Groenland et l'efficacité phagocytaire chez les nouveau-nés de phoques gris étaient significativement plus élevés que chez leurs mères. Les résultats pourraient refléter la capacité de phagocytose des nouveau-nés de phoques gris et du Groenland à répondre aux pathogènes. Les résultats de cette étude suggèrent que la phagocytose chez les différentes espèces de phoques examinées n'est pas totalement développée à la

naissance puisqu'elle augmente chez les nouveau-nés durant la lactation. De plus, l'activité phagocytaire des phoques semble diminuer au cours de la vie chez l'adulte.

Veterinary Immunology and Immunopathology xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Phagocytosis in pup and adult harbour, grey and harp seals

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ARTICLE INFO

Article history:
Received 20 March 2009
Received in revised form 21 August 2009
Accepted 24 August 2009

Keywords:
Phagocytosis
Harbour seals
Grey seals
Harp seals
Lactation

ABSTRACT

Knowledge on pinniped immunology is still in its infancy. For instance, age-related and developmental aspects of the immune system in pinnipeds need to be better described. The present study examined the phagocytic activity and efficiency of harbour, grey and harp seal leukocytes. In the first part of the study, peripheral blood was collected from captive female harbour seals of various ages. Data showed an age-related decrease in phagocytosis in female harbour seals from sub-adult to adulthood. In the second part of the study, changes in phagocytosis were quantified during lactation in wild newborn harbour, grey and harp seals and in their mothers (harp and grey seals). In newborns of the same age, leukocytes of harbour and harp seals phagocytosed less than those of grey seal pups. The phagocytic activity and efficiency increased significantly from early to mid-lactation in newborn harbour seals, and from early to late lactation in newborn grey seals, which could suggest that the transfer of phagocytosis-promoting factor(s) in colostrum is an important feature of temporary protection for pups. In contrast, no changes in phagocytic activity and efficiency were observed in lactating females of the two seal species, harp and grey, examined. At late lactation, phagocytic activity in both grey and harp seal pups and phagocytic efficiency in grey seal pups were significantly higher than in their mothers. These results could reflect either the capacity of phagocytes of the newborn harp and grey seals to respond to pathogens. Results from this study suggest that the phagocytosis of the seal species examined is not fully developed at birth as it generally increases in pups during lactation. Thereafter, the phagocytic activity of seals appears to decrease throughout adulthood.

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1. Introduction

The immune system uses a complex network of interacting cells and chemical messengers to mount rapid, specific, protective responses against the foreign pathogens or to prevent the growth of malignant tissues. Although comparative studies have found that some differences exist between the immune system of pinnipeds and that of other mammals, most fundamental immunological features, such as immunological effector cells and immunoglobulins, are similar (Cavagnolo, 1979; Ross et al., 1993).

Critical to the long-term survival of a given species is the efficiency with which newborn animals cope with pathogens they encounter in their environment. As suggested by Ross and De Guise (2007) developmental immunology of marine mammals exhibit a number of species-specific adaptations with respect to their habitat needs. Adaptations include maternal care, duration of gestation and nursing periods, which figure prominently in the ontogeny of the immune system in marine mammals (Ross et al., 1994). Newborn harbour seals appear to have a relatively competent immune system as demonstrated with mitogen-induced lymphocyte proliferation and

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doi:10.1016/j.vetimm.2009.08.017

Please cite this article in press as: Frouin, H., et al., Phagocytosis in pup and adult harbour, grey and harp seals. Vet. Immunol. Immunopathol. (2009), doi:10.1016/j.vetimm.2009.08.017

antibody response to rabies immunisation (Ross et al., 1993, 1994). In both harbour seal (Ross et al., 1993, 1994) and grey seal (King et al., 1994) immunoglobulin levels were low at birth and steadily increased during early life. As a temporary protection, pups receive maternal antibodies via colostrum (King et al., 1994; Ross et al., 1993, 1994). Immune function studies conducted on pinnipeds, mostly harbour seals, identified methods which proved suitable for the assessment of non-specific immune function in both captive (De Swart et al., 1993; King et al., 1993) and field (Ross et al., 1993, 1994) studies of harbour seals.

The phagocyte system is an essential component of innate immunity, particularly in complex metazoans where specialized phagocytes (macrophages and neutrophils) perform various host defence functions that rely on the phagocytic uptake of pathogens. Phagocytosis plays a key role in innate (non-specific) immune responses of mammals and represents the principal effector mechanism for the ultimate disposal of neoplastic cells or microbes (Van Oss, 1986). Neutrophils are the most important circulating phagocytes, providing the first line of defence against invading particles, especially bacteria (Van Oss, 1986). Circulating monocytes, precursors of tissue macrophages, also have the ability to phagocytose (Cline and Lehrer, 1968). The phagocytic activity of beluga (*Delphinapterus leucas*) peripheral blood granulocytes has been used as an indicator of the status of non-specific immunity (De Guise et al., 1995). In studies on toxic effects of exposure to xenobiotics, phagocytosis has been identified as useful tool in lab-based studies to assess the immune competence of leukocytes of marine mammals such dolphins, whales or seals exposed *in vitro* to pollutants (Camara Pellisso et al., 2008; De Guise et al., 1998; Frouin et al., 2008).

The St. Lawrence marine ecosystem (SLME) that includes the St. Lawrence Estuary and the Gulf of St. Lawrence is inhabited by several species of seals. These include harbour seals (*Phoca vitulina*), grey seals (*Halichoerus grypus*) and harp seals (*Phoca groenlandica*). Harbour seals are distributed throughout the study area, occur in several colonies and they are permanent residents of the Estuary and Gulf of St. Lawrence (Lebeuf et al., 2003). Grey seals may summer in the St. Lawrence Estuary, but over-winter in the Gulf of St. Lawrence or on the Atlantic coast of Canada (Lavigeur and Hammill, 1993). Harp seals over-winter in the SLME, but summer in Arctic waters (Stenson and Sjare, 1997).

Several biological differences exist between the harbour, grey and harp seals. For instance, the average lactation periods for harbour, grey and harp seals are 24, 16 and 12 days respectively (Bowen, 1991; Bowen et al., 1992a). Moreover, harp seals are pack-ice breeders (Lavigne and Kovacs, 1988), harbour seals are land breeders and in Canada, grey seals are both land and ice breeders (Schulz and Bowen, 2004).

The present study focuses on the phagocytic activity and efficiency in three phocids. The main objective of this work was to investigate the effects of age on the evolution of phagocytosis in seals. The first part of the study examined the phagocytic activity and efficiency in female harbour seals from sub-adult to adulthood. The second part of the study focused on the lactation period and examined (1) the development of phagocytic activity and efficiency in newborn harp, grey and harbour seals and (2) the evolution of phagocytosis in lactating grey and harp seal females.

2. Materials and methods

2.1. Animal capture, handling, and sample collection

Seals were captured and sampled in the Gulf of St. Lawrence in 2008 (Tables 1 and 2; Fig. 1). Blood was obtained from eight live captured young of the year (pup) harbour seals in Newfoundland in May of 2008 (Fig. 1). Seals were captured in the water using a dip net and an inflatable boat, and subsequently transferred to a larger boat where all handling took place. Prior to analysis, seals were weighed (to ±0.5 kg), tagged, and their sex noted (Dubé et al., 2003). All handling of the pup was carried out as quickly as possible to avoid abandonment of the pup by the mother (Boulva and McLaren, 1979). Pups were captured throughout the lactation period. The blood samples, up to 50 ml, were taken from the extradural intravertebral vein into Vacutainer tubes containing sodium heparin as anticoagulant (Becton-Dickinson, NJ, USA) and were immediately stored between 5 °C and 15 °C, and processed later (~6 h after sampling) in a field laboratory.

Eighteen grey seal and seventeen harp seal mother–pup pairs respectively were live-captured in early January of 2008 near Amet Island in the Northumberland Strait and on Hay Island off the east coast of Cape Breton Island and on the pack-ice near the Magdalen Islands in the Gulf of St Lawrence in March 2008 (Fig. 1; Tables 1 and 2). Pairs were captured on the ice using nets and blood was drawn from

Table 1
Species, sampling location, date and characteristics of pup phocids sampled in the Gulf of St Lawrence.

Species	Sampling location		n	Sex	Age (days)	Body condition	Isolation of PBL (peripheral blood leukocytes)	Collection date (date/month/year)
	Sector	Region						
Harbour seals	Newfoundland	Gulf	8	3F, 5M	2–11	71 ± 3.2 (63.2–84.4)	Erythrocyte lysis	24–26/05/2008
Grey seals	Amet Island and Hay Island	Gulf	18	12F, 6M	0–14	75.9 ± 2.1 (66.7–85.6)	Erythrocyte lysis	12, 24–25/01/2008
Harp seals	Magdalen Islands	Gulf	17	12F, 5M	0–10	84.6 ± 2 (72.7–103.4)	Erythrocyte lysis	6–7, 10/03/2008

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Table 2
Species, sampling location and date of adult phocids sampled in Zoo and Gulf of St. Lawrence.

Species	Sampling location		n	Sex	Age (years)	Isolation of PBL (peripheral blood leukocytes)	Collection date (date/month/year)
	Sector	Region					
Harbour seals	Aquarium (Québec)	n.a.	4	4F	10–18	Lympholyte Mammal	05/11/2008
	Zoo (St Félicien)	n.a	4	4F	3–31	Lympholyte Mammal	18/06/2008
Grey seals	Amet Island and Hay Island	Gulf	18	18F	n.d.	Erythrocyte lysis	12, 24–25/01/2008
Harp seals	Magdalen Islands	Gulf	17	17F	n.d.	Erythrocyte lysis	6–7, 10/03/2008

n.a.: not applicable; n.d.: not determined.

the extradural intravertebral vein using an 18-gauge needle mounted on a 50-ml syringe for adult seals or a 20-gauge needle and a 50-ml syringe for pups. The blood samples, up to 50 ml, were transferred into Vacutainer tubes (Becton-Dickinson, NJ, USA) and were immediately stored between 5 °C and 15 °C, and processed later (~6 h after sampling) in a field laboratory. The volume of blood collected was no more than 10% of the total blood volume (McGuill and Rowan, 1989). All research was approved by the Animal Care Committees of Fisheries and Oceans, Canada.

Body condition was assessed by calculating a condition factor for each seal (axillary girth divided by standard length, multiplied by 100; McLaren, 1958). Pup seals were aged by mass. Newborn pups were identified by the presence of fresh placentas and blood on the ice. Age was estimated from the total body weight of the pup according to the formulas previously determined and specific for each species:

- harp seal. We used the equation of Stewart and Lavigne (1980): age (days) = 0.35 × body weight (kg) − 3.2.
- grey seal. We used the equation age (days) = [body weight (kg) − 16]/2.8 determinate from Bowen et al. (1992b).
- harbour seal. We used the equation derived from Dubé et al. (2003): age (days) = [body weight (kg) − 11.4]/0.54.

Pups weighing less than the mean mass at birth (value in formula) at the time of capture were considered to be newborns (day 0).

Fresh blood samples were also collected in 2008 from 8 known-age female harbour seals housed in aquaria (Aquarium de Québec, Zoo de Saint-Félicien) (Table 2). These samples were taken from a superficial plantar flipper veins and approximately 10–40 ml of whole bloods was collected from each animal. Blood was kept at 4 °C until the time of analysis, 6–8 h after sampling.

2.2. Isolation of peripheral blood leukocytes (PBL)

In the present study, isolation of PBL was obtained by two different techniques, either by Lympholyte® separation or whole blood lysis. Both methods allow the removal of debris, dead cells and erythrocytes and the retaining of the monocytes and neutrophils with the lymphocytes. When access to laboratories was not limited, cells were isolated by Lympholyte® gradient purification in the laboratory, otherwise cells were isolated only by erythrocyte lysis in the field. Data obtained from erythrocyte lysis

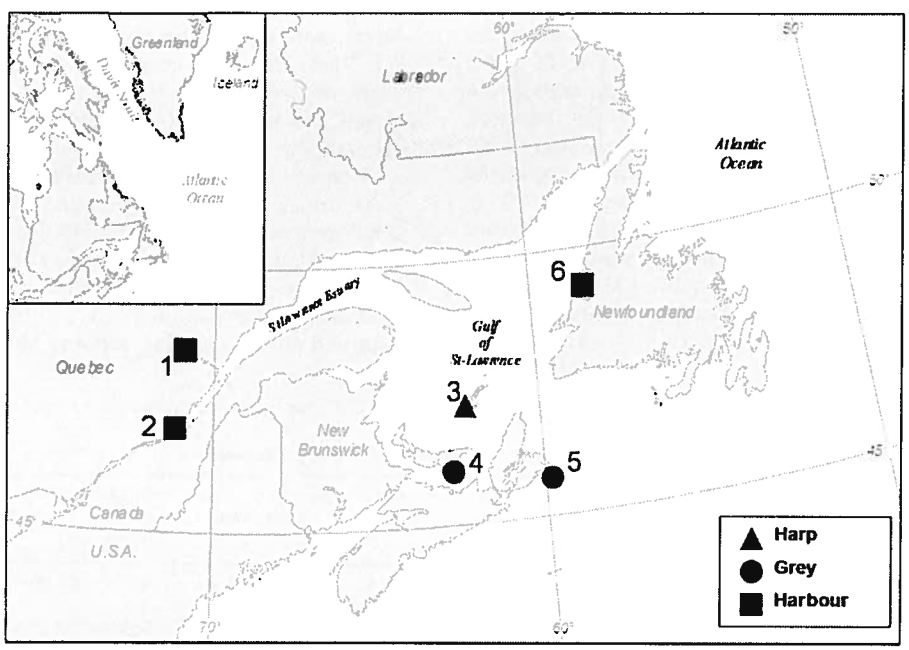

Fig. 1. Location of capture site for pup seals collected in the Gulf of St. Lawrence and zoo facilities. (1) Zoo (St. Félicien); (2) Aquarium (Quebec); (3) Madgalen Islands; (4) Amet Island; (5) Hay Island; (6) Newfoundland.

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were not combined with data obtained from gradient density. They represent two separate studies, one to determine the influence of age on phagocytosis in harbour seals (PBL isolated by Lympholyte®) and the other to evaluate the evolution of phagocytosis during lactation period in pup and mother seals (PBL isolated by erythrocyte lysis).

2.2.1. Lympholyte mammal

The blood from seals housed in zoo facilities was diluted 1:1 in Phosphate Buffer Saline (PBS); the cellular suspension was then carefully laid on a lympholyte-mammal solution (1.086 g/ml; Cedarlane Laboratories Ltd., Hornby, Canada). The leukocytes were then isolated by centrifugation for 20 min at 800 × g. The PBL layer was washed three times with PBS at 800 g and resuspended in RPMI-1640 (Sigma) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 1% Pen-Strep and 10 mM Hepes (complete medium). The ratio of live/dead cells was assessed using trypan blue dye exclusion and visual examination under a light microscope with a hemacytometer. The cells were kept overnight at 4 °C and viability was assessed a second time before analysis. Cell viability was always greater than 90%.

2.2.2. Erythrocyte lysis

Whole blood from wildlife seals was diluted 1:17 with an aqueous solution containing 9 g/l NH_4Cl (Sigma), 1 g/l $KHCO_3$ (Sigma) and 37 mg/l tetrasodium salt EDTA (Sigma) at 37 °C for 7 min to ensure red cell lysis by osmotic shock. After three washes with PBS, the ratio of live/dead cells was assessed microscopically using trypan blue dye exclusion and a hemocytometer. This ratio was always greater than 90%. The cells were used immediately.

2.3. Phagocytosis

The cell concentration was adjusted to 10^6 viable PBL in complete medium. Phagocytic activity was measured, based on the protocol of Brousseau et al. (1999). Briefly, 1.719 μm diameter yellow green fluorescent latex beads (Molecular Probe Inc., Eugene, OR) were added to each cell suspension at a ratio of 100 beads per leukocyte and incubated at 37 °C in a shaker for 1 h. Cells were separated from non-phagocytised beads by centrifugation for 8 min at 150 g (4 °C) on a cushion of 3% bovine serum albumin (BSA, Fraction V) in RPMI-1640 supplemented with 10% FCS. The supernatant was discarded and cells were then resuspended in an isotonic solution (Hematall; Becton Dickinson). Samples were analysed with a FACScalibur flow cytometer (Becton Dickinson, San José, USA) equipped with a 488 nm argon laser. For each sample, the fluorescence of 10,000 events was recorded. Results were analysed with the Cell Quest Pro software (Becton Dickinson) to determine the percentage of PBLs (monocytes and neutrophils) that engulfed one bead and more (phagocytic activity) or three beads and more (phagocytic efficiency) (Fig. 2). The fluorescent response of lymphocytes was used as negative controls to evaluate the surface attachment of beads that were not processed by phagocytic activity (non-specific attachment) (Fig. 2). All

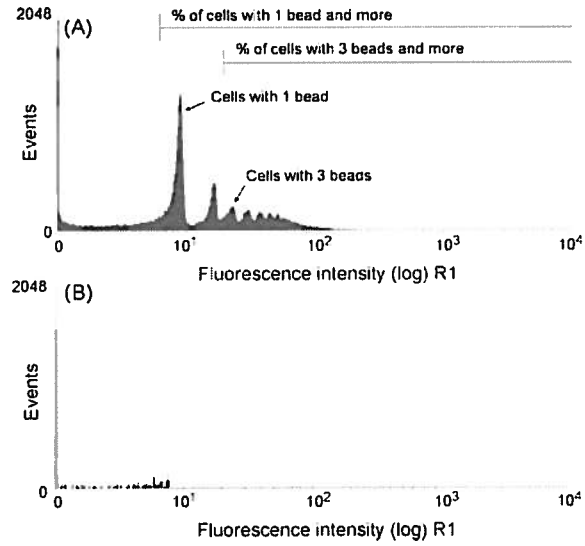

Fig. 2. Representative fluorescence histogram of harbour seal neutrophils after phagocytosis of fluorescent latex beads by PBLs (A). Free beads were used as a reference. Phagocytic activity is defined as the percentage of cells that phagocytised one bead and more whereas phagocytic efficiency is defined as the percentage of cells that phagocytised three beads and more. The absence of fluorescent response observed in lymphocytes (B) corresponds to their inability to perform phagocytosis and represents an appropriate negative control.

samples were measured in duplicate; mean values were used for subsequent analysis. The results were expressed as the percentage of phagocytosis (neutrophils and monocytes combined).

2.4. Statistical tests

All statistical analyses were undertaken using STATISTICA for windows (version 7.0, StatSoft Inc., 1995). Linear regression analysis between phagocytosis performance and age in female harbour seals, day post-parturition (dpp) in harbour, grey and harp seal pups and day post-parturition in lactating grey and harp seals were performed by *t*-test for significance of fit (H0: slope = 0). This analysis determines if age or day post-parturition contributes to predicting the dependent variable (phagocytosis). Linear regression analysis between phagocytosis performance and body condition in harbour, grey and harp seal pups was performed by *t*-test for significance of fit (H0: slope = 0). This analysis determines if body condition contributes to predicting the dependent variable (phagocytosis).

Differences of phagocytic activity and efficiency and differences of body condition between harbour, grey and harp seal pups were investigated using a single factor analysis of variance (ANOVA) and, when significant, a Tukey's honest significant difference test was used post hoc to determine which group differed. A p value of less than 0.05 was considered to have statistical significance.

Student's *t*-tests were performed to identify potential differences for phagocytic activity and efficiency in harbour, grey and harp seal pups between early lactation (0–5 dpp) and late lactation (6–14 dpp). Student's *t*-tests

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were performed to identify potential differences for phagocytic activity and efficiency between lactating grey seals and lactating harp seals and to identify potential differences between phagocytic activity and efficiency of pups versus their mothers at early lactation (0–5 dpp) and late lactation (6–14 dpp) for each seal species (harp and grey seals). A p value of less than 0.05 was considered to have statistical significance.

3. Results

3.1. Regression of phagocytic activity with age in harbour seals

The regression between phagocytic activity and age was evaluated in PBL, isolated with Lympholyte®, from blood collected in 2008 from eight female captive harbour seals from two zoo facilities. A significant negative correlation was noted between increasing age and phagocytic activity in females ($r^2 = -0.89$; $p < 0.001$) (Fig. 3).

3.2. Phagocytic activity and efficiency of the newborn harbour, grey and harp seals during lactation

Peripheral blood leukocytes were isolated with erythrocyte lysis. The immune response in newborn harbour, grey and harp seals was examined during lactation (Fig. 4). Age of seals had a significant effect on phagocytic activity and efficiency in harbour and grey seals. A significant correlation was noted between increasing day post-parturition and phagocytosis activity in harbour ($r^2 = 0.60$; $p = 0.03$) and grey ($r^2 = 0.58$; $p < 0.001$) seal pups and phagocytic efficiency in harbour ($r^2 = 0.89$; $p < 0.001$) and grey ($r^2 = 0.62$; $p < 0.001$) seal pups. In harp seal pups, no correlation was found between day post-parturition and phagocytic activity ($r^2 = 0.10$; $p = 0.20$) and efficiency ($r^2 = 0.01$; $p = 0.73$). Phagocytic activity and efficiency of early lactation (0–5 dpp) were

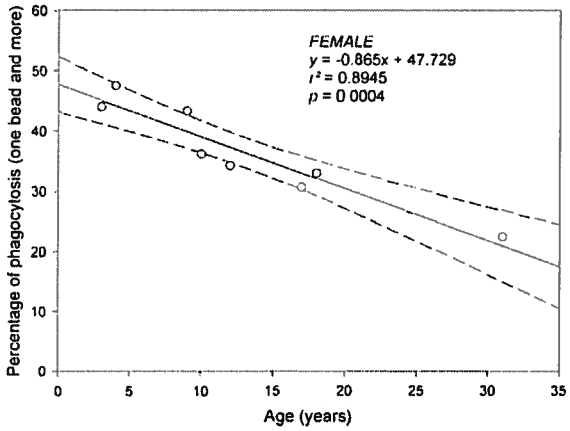

Fig. 3. Regression analysis of phagocytic activity (percentage of phagocytes having engulfed one bead and more) versus age of female harbour seals. The regression line (solid line) and the 95% confidence intervals (dashed lines) are shown. A significant negative correlation is noted $R = -0.89$.

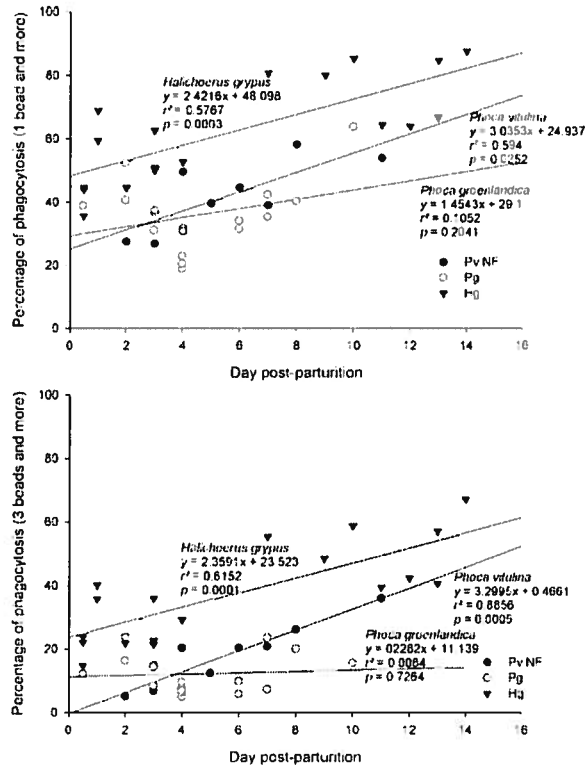

Fig. 4. Phagocytic activity (percentage of phagocytes having engulfed one bead and more) and efficiency (percentage of phagocytes having engulfed three beads and more) during lactation in pups of harp, grey and harbour seals.

compared to those of mid/late lactation (6–14 dpp) in newborn harbour, grey and harp seals. In newborn grey seals, the phagocytic activity ($p < 0.001$) and efficiency ($p < 0.001$) increased significantly from early (0–5 dpp) to late lactation (6–14 dpp). In newborn harbour seals, the phagocytic activity ($p = 0.048$) and efficiency ($p = 0.046$) increased significantly from early- (0–5 dpp) to mid-lactation (6–14 dpp). The phagocytosis rate did not increase significantly during lactation period in harp seals. However, we observed a significant difference in phagocytic activity at early lactation (0–5 dpp) between grey and harbour seal pups (ANOVA, $p = 0.002$) and between grey and harp seal pups (ANOVA, $p < 0.001$). A significant difference in phagocytic efficiency was observed between grey and harbour seal pups (ANOVA, $p = 0.003$) and between grey and harp seal pups (ANOVA, $p < 0.001$) at early lactation (0–5 dpp). At mid/late lactation (6–14 dpp), a significant difference in phagocytic activity was obtained between grey and harbour seal pups (ANOVA, $p < 0.001$) and between grey and harp seal pups (ANOVA, $p < 0.001$). Finally, a significant difference in phagocytic efficiency was observed between grey and harbour seal pups (ANOVA, $p < 0.001$) and between grey and harp seal pups (ANOVA, $p < 0.001$) at mid/late lactation (6–14 dpp).

No correlation was found between body condition and phagocytic activity in harp seal pups ($r^2 = 0.07$; $p = 0.320$), in harbour seal pups ($r^2 = 0.03$; $p = 0.689$) and in grey seal pups ($r^2 = 0.02$; $p = 0.735$). Moreover, no correlation was

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found between body condition and phagocytic efficiency in harp seal pups ($r^2 = -0.07$; $p = 0.801$), in harbour seal pups ($r^2 = 0.01$; $p = 0.958$) and in grey seal pups ($r^2 = 0.02$; $p = 0.728$). However, the body condition was statistically different between species (ANOVA, $p = 0.014$). A Tukey's post hoc comparison revealed that body condition was similar between harp and grey seal pups and between grey and harbour seal pups but statistically different between harp and harbour seal pups ($p = 0.011$).

3.3. Phagocytic activity and efficiency in free-ranging harp and grey seal mothers during lactation

Peripheral blood leukocytes were isolated with erythrocyte lysis. No changes in phagocytic activity and efficiency were observed in harp or grey seal mothers during lactation (Fig. 5). No correlation was found between day post-parturition and phagocytic activity ($r^2 = 0.16$; $p = 0.11$; $r^2 = 0.13$; $p = 0.16$) and efficiency ($r^2 = 0.04$; $p = 0.43$; $r^2 = 0.22$; $p = 0.06$) in grey and harp seal mothers respectively. However, lactating grey seals have a higher phagocytic activity ($p < 0.001$) and efficiency ($p < 0.001$) than those of lactating harp seals.

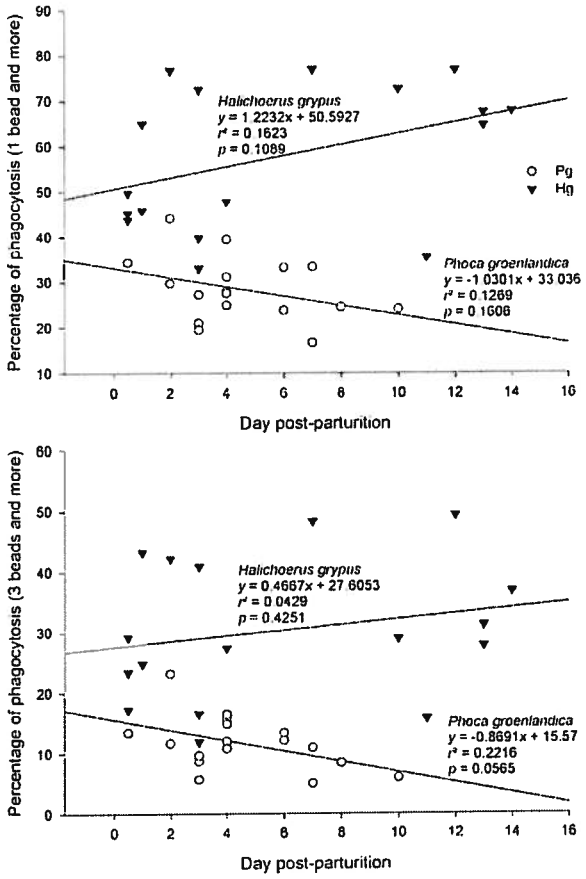

Fig. 5. Phagocytic activity (percentage of phagocytes having engulfed one bead and more) and efficiency (percentage of phagocytes having engulfed three beads and more) in harp seal and grey seal mothers throughout the lactation period.

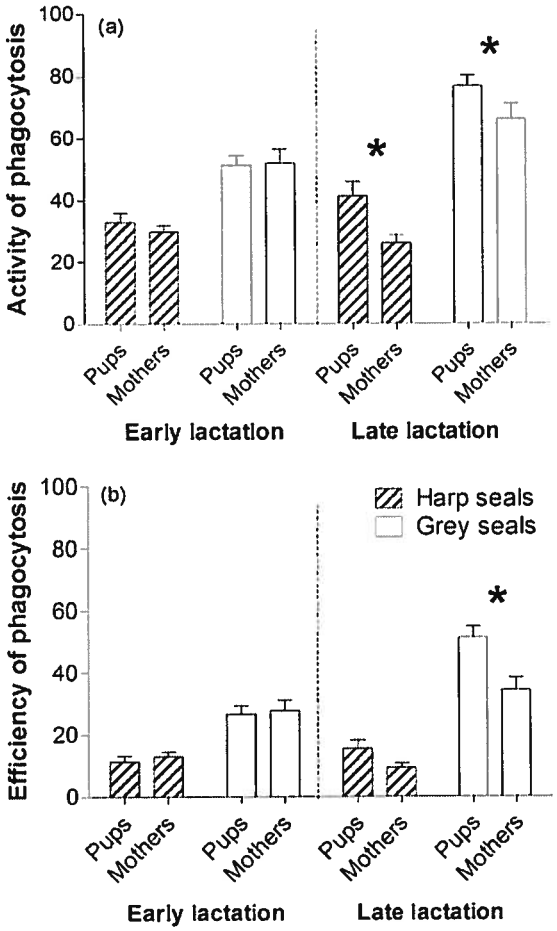

Fig. 6. Mean (±SE) phagocytic activity (a) and efficiency (b) of PBL from pup and mother harp seals (hatched bars; $n = 11$ at early lactation; $n = 6$ at late lactation) and from pup and mother grey seals (open bars; $n = 10$ at early lactation; $n = 8$ at late lactation). Early lactation is 0–5 days after parturition, late parturition is 6–14 days after parturition. Significant differences between mothers and pups for time points are indicated by *($p < 0.05$), as measured by t-test.

3.4. Phagocytic activity and efficiency in pups versus their mothers

Peripheral blood leukocytes were isolated with erythrocyte lysis. In both harp and grey seals, early in the lactation period phagocytic activity and efficiency of pups were similar to those of their mothers (Fig. 6). During the late lactation period the phagocytic activity ($p = 0.048$) and efficiency ($p < 0.001$) were higher in the pup grey seals than those in their mothers. In harp seals a significant difference between pups and mothers was observed at late lactation only for phagocytic activity ($p = 0.038$).

4. Discussion

In this study, phagocytic activity and efficiency were evaluated as a function of age in pup and adult harp, harbour and grey seals.

The present work demonstrates that phagocytic activity in elderly female seals was diminished. However, in the

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literature, the effects of age on phagocytosis by polymorphonuclear leukocytes (PMN) for adult organisms are varied. Several studies found in humans an age-related decline of PMN phagocytic activity due to a decreased number of PMNs that are able to ingest target beads (Nagel et al., 1986) or due to a reduced chemotaxis (Wenisch et al., 2000). However, other authors found no age effect on phagocytosis of human PMNs (Krause et al., 1999) and suggested that nutritional and health status should be considered in tandem when examining the effects of aging on immune function, as an impact of nutritional status on immune function is possible. The finding of the present study supports an age-related decline in phagocytic activity in adult female harbour seals. As these seals were apparently healthy and well nourished, it is assumed that the nutritional/health status did not account for changes in phagocytosis and was most likely associated to aging. Moreover, Trumble et al. (2006) has previously demonstrated that immune parameters evaluated by the haematological profiles in captive harbour seals were not affected by diet. Nielsen (1995) has shown that several parameters of the immune system in captive harbour seals decreased with age including erythrocyte-rosette (E-rosette) test and lymphocyte transformation test (LTT). They also demonstrated the decline of the percentage of lymphocytes and the corresponding increase in neutrophils with age, whereas no relationship existed between the percentages of monocytes and eosinophils and age (Nielsen, 1995). Thus, the observed age-related decrease in phagocytosis in female harbour seals may not be explained by a decreased number of phagocytes (neutrophils and monocytes) but might be associated to a reduced chemotaxis, which corresponds to previous findings in human (Niwa et al., 1989; Wenisch et al., 2000).

To our knowledge, this is the first study on the phagocytosis of immune cells from peripheral blood of seals during the lactation period. This study showed that the innate immune response in neonates differs both qualitatively and quantitatively among species. During the lactation period, phagocytic activity and efficiency statistically increased with age in grey and harbour pup seals but not in harp seals. The increasing phagocytic activity and efficiency reported in newborns of this study contrast with the observation that the mouse macrophage function appeared to be impaired in the neonate, possibly as a result of inhibitory factors present in the serum (McKay and Lu, 1991). However, phagocytosis-promoting factor(s) are present in human and bovine colostrum (Straussberg et al., 1995; Menge et al., 1998). It appears that the phagocytosis-promoting activity in colostrum peaks at parturition and that both its activity and the level of colostral immunoglobulin G (IgG) gradually declines in bovine colostral milk (Sugisawa et al., 2001). This phenomenon suggests that the colostral IgG act as the phagocytosis-promoting factor. Furthermore, it has been shown that a transfer of maternal IgG to pups through colostrum occurred in harbour and grey seals (Carter et al., 1990; Ross et al., 1993, 1994) with an increase in pup IgG levels during the nursing period (Ross et al., 1994; Carter et al., 1990).

The elevated phagocytosis observed in pup grey seals compared with those in harp and harbour seals might be related to differences in body condition (Young, 1976; Chandra, 1993). Furthermore, pathogen exposure and interaction with the immune system might influence phagocytosis. Fowler (1990) demonstrated that disease-cause mortality in fur seal pups was dependent of the pup density on land. Land-breeding seals often occur in very large aggregations during the breeding season (Hindell, 2002), whereas, ice is not a limited resource, and females can haul out anywhere to breed, so aggregations of females tend not to occur (Hindell, 2002). Therefore, the lower phagocytic activities of ice-breeding harp seals may have been a result of a low exposure to pathogens comparatively to land-breeding counterparts. However, influence of other environmental factors including stress (Romano et al., 2002), xenobiotics (Ross et al., 1996) and temperature (Bossart et al., 2002) cannot be excluded. Therefore, the differences observed between the species of this study could be not only due to inter species differences, but also due to differences in the pathogens and/or environmental factors that they are exposed to at birth.

Interestingly, similar to results found in the pups, the lactating grey seals revealed higher phagocytic activity and efficiency than lactating harp seals. Recent studies have been carried out on variations in major histocompatibility complex (MHC) class II alleles in several seal species. These studies revealed possible inter-specific variation to infectious diseases in four Antarctic phocid species (Lehman et al., 2004) and considerable MHC variation in northern elephant seals (*Mirounga angustirostris*) and southern elephant seals (*Mirounga leonina*) (Hoelzel et al., 1999). Therefore, interspecies differences in immune functions are very likely and might be cause of the differences detected in this study.

The phagocytosis of harp and grey seal pups was similar to or higher than that of their mothers reflecting the capacity of phagocytes of newborn harp and grey seals to respond to pathogens. The apparent immune competence of pups may reflect a combination of adaptations to a short nursing period, limited maternal care (Kovacs and Lavigne, 1986) and the extent to which the host encounters bacteria and other pathogens in the environment.

Limitations of this study were inherent to all studies with wide-life animals. Only a small number of animals were present in the different groups, which might have affected statistical power and results of female and male animals were not separated. However, the results of this study revealed age-related changes of the immune system of pinnipeds. Whereas newborn pups showed increasing phagocytic activity and efficiency during the lactation period, indicating immune competence, adult seals revealed a decrease in phagocytic activity during adulthood.

5. Conclusion

Newborn harbour, harp and grey seals revealed similar phagocytic activity and efficiency between species and with their mothers, suggesting a competent immune system. The increase in phagocytosis through the nursing period suggests that the transfer of phagocytosis-promot-

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ing factor(s) in colostrum obtained early in lactation might be an important aspect of temporary immunological defence. In adult seals, the phagocytic activity decreases throughout their lifetime, as seen in harbour seals. Further investigations of different parameters of the immune system (humoral/cellular), long-term studies and identification of parameters/factors impacting the immune system in pinnipeds are needed.

Acknowledgements

This work is part of the Research Programme undertaken by the Canada Research Chair in Environmental Immunotoxicology (M.F.). This research project was funded in part by the Department of Fisheries and Oceans (M.L. and M.H.). We thank everyone who took part in planning and field assistance, B. Sjare, S. Turgeon, S. Trottier, H. MacRae, E. Chassot, J.-F. Gosselin, Y. Dubé, W. Penney. The expert technical assistance of Marlene Fortier was greatly appreciated. The authors wish to thank the staffs of the aquarium du Québec (S. Masson/Dr. Chantal Proulx) and the zoo at St.-Félicien (C. Gagnon) for providing blood samples from the marine mammals.

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3.2. Les réponses proliératives des lymphocytes T d'un phoque gris (*Halichoerus grypus*) exposés aux métaux lourds et aux BPCs *in vitro*

Article 2 : *Héloïse Frouin, Lucie Ménard, Lena Measures, Pauline Brousseau, Michel Fournier. T lymphocyte-proliferative responses of a grey seal (Halichoerus grypus) exposed to heavy metals and PCBs in vitro.*

Mon rôle a consisté à compiler les données brutes de l'étude, les analyser, les interpréter et écrire cet article à titre d'auteur principal.

RÉSUMÉ

Cette étude analysait les effets du chlorure de methylmercure (CH_3HgCl), du chlorure de zinc ($ZnCl_2$), du chlorure de cadmium ($CdCl_2$), de l'acétate de plomb ($Pb(C_2H_3O_2)_2$) et des BPC (mélanges d'Aroclor) sur la prolifération des lymphocytes T *in vitro*, en utilisant les cellules isolées du thymus, d'un ganglion lymphatique et du sang d'une jeune femelle phoque gris (*Halichoerus grypus*). Suite à l'exposition aux métaux lourds ou aux mélanges Aroclors une courbe dose-réponse est observée avec une diminution de la prolifération des lymphocytes T du thymus, du ganglion lymphatique et du sang périphérique. Ces résultats démontrent la sensibilité des lymphocytes T de phoque gris au CH_3HgCl et au $ZnCl_2$ présents dans leur environnement à des concentrations trouvées dans leurs sang, Si les lymphocytes de n'importe quel phoque gris se comporte *in vivo* comme ils se comportent *in vitro* dans cette étude, alors, la capacité diminuée des lymphocytes à proliférer suite à une exposition aux métaux lourds et aux PCB, pourrait mener à l'incapacité à fournir une réponse immunitaire adéquate chez les phoques gris.

ABSTRACT

This study investigated *in vitro* the effects of methylmercury chloride (CH_3HgCl), zinc chloride ($ZnCl_2$), cadmium chloride ($CdCl_2$), lead acetate ($Pb(C_2H_3O_2)_2$) and PCBs (Aroclor mixtures) on proliferation of T lymphocytes from the thymus, lymph node and blood from one female grey seal (*Halichoerus grypus*) juvenile. After exposure to heavy metals or Aroclor mixtures a dose-response curve was observed with a decrease in proliferation of T lymphocytes from thymus, lymph node and blood. These results demonstrate the sensitivity of grey seal T lymphocytes to CH_3HgCl and $ZnCl_2$ present in their environment at concentrations that are found in their blood. If lymphocytes from any grey seal behave *in vivo* as they do *in vitro* in this study, then, the altered ability of lymphocytes to proliferate post-exposure to heavy metals and PCBs might lead to the inhability to mount an adequate immune response in grey seals.
Keywords : heavy metals; PCBs; grey seal; immune tissues; immunotoxicity; *Halichoerus grypus*

INTRODUCTION

Several studies have investigated the effect of contaminant exposure and uptake in marine mammals, particularly pinnipeds (Helle et al., 1976; Reijnders, 1986). Effects on immune function have been inferred from associations between organochlorine contaminant levels in the blubber or blood and changes in immune blood parameters (Beckmen et al., 2003) or from associations with disease. An experimental semi-field study in harbour seals demonstrated a suppression of lymphocyte proliferation in the organochlorine-exposed group (De Swart et al., 1994). Moreover, there is an increasing evidence that environmental contaminants, and PCBs in particular, may have played a role in the severity of morbillivirus-induced mass mortalities among harbour seal population in 1988 (Ross et al., 1995).

Possible immunosuppression by other groups of environmental contaminants, such as heavy metals, may occur. Iimmunotoxic effects of cadmium has not been well studied in marine mammals (De Guise et al., 1996). Bennett et al. (2001) found that zinc was significantly higher in harbour porpoises (*Phocoena phocoena*) that died of infectious disease compared to healthy porpoises that died from physical trauma. While inorganic mercury is the major source of Hg in most aquatic systems, it is methylmercury which bioaccumulates and biomagnifies at all trophic levels in marine food webs (Gray, 2002) and which is the contaminant associated with severe toxic effects in wildlife

(reviewed byWolfe et al., 1998). *In vitro* exposure to mercury impairs specific immune defenses of many species including marine mammals (Camara Pellisso et al., 2008; De Guise et al., 1996; Lalancette et al., 2003; Pillet et al., 2000). In contrast, exposure to lead did not affect proliferation of lymphocytes from beluga (*Delphinapterus leucas*) in vitro (De Guise et al., 1996).

Despite the growing body of literature on immunotoxic effects in harbour seals (*Phoca vitulina*), far less work has been carried out on grey seals. Few studies have investigated the effects *in vitro* of organic contaminants (Hammond et al., 2005) and heavy metals (Lalancette et al., 2003; Pillet et al., 2000) on grey seals. Many environmental toxicants with a potential toxicity to the hematopoietic system have been identified by animal experiments. Owing to the risks of severe chronic hematopoietic disorders, it is important to screen chemicals for their hematotoxicity (Van Den Heuvel et al., 2001). The response of immune cells from different organs or tissues has not been investigated in grey seal.

In order to be able to measure possible deleterious effects of environmental contaminants on the immune system of pinnipeds, a logical first step is to evaluate the proliferation of lymphocytes from different immune tissues exposed to contaminants. The purpose of the present study is to evaluate whether the mitogen-induced proliferative responses of T lymphocytes from thymus, blood and lymph node from the grey seal are altered after chemical exposure *in vitro*.

MATERIAL AND METHODS

In an experimental study on the biology of lungworm, *Otostrongylus circumlitus*, (L. Measures unpublished data), female grey seal pups born and weaned naturally after 2-wk lactation were captured in the Gulf of St. Lawrence in February 2000. These 3-wk old animals were brought to the Maurice-Lamontagne Institute (Mont-Joli, Quebec) and held in captivity under similar husbandry as described in Gajadhar et al. (2004). Grey seals were cared for according to the Canadian Council on Animal Care with an experimental protocol approved by the Animal Protection Committee at the Maurice-Lamontagne Institute. Seals were exposed to *O. circumlitus* infective larvae and killed at intervals to follow development. One seal approximately four month old and weighting 58.5 kg, was euthanized by injection of Euthanyl ® (MTC Pharmaceutical, Cambridge, Ontario) 8-d post-exposure. Before euthanasia, a blood sample, around 100 ml, was collected using heparinized Vacutainer tubes (Becton-Dickinson, NJ, US) and kept at

4℃ until further processing and analysis within 6 hours post-sampling. At necropsy, no worms were observed, and a thoracic lymph node and the thymus were collected and kept at 4℃ in culture medium (RPMI 1640; Gibco BRL, Mississauga, Ontario, Canada) supplemented with 10% fetal calf serum (Gibco BRL), 100 U penicillin (Gibco BRL) and 10 mM Hepes (Gibco BRL), until further processing and analysis within 6 hours post-sampling. We assumed that no stress associated with captivity nor the failure to infect this seal with *O. circumlitus* affected the seal's susceptibility to heavy metals and PCBs.

Cadmium chloride ($CdCl_2$) and zinc chloride ($ZnCl_2$) were purchased from Sigma Chemicals (Oakville, Ontario, Canada). Lead acetate ($Pb(C_2H_3O_2)_2$) was purchased from Fisher (Montreal, Quebec, Canada). Stock solutions were prepared in distilled water at a concentration of 10^{-2} M. Methylmercuric chloride (CH_3HgCl) was obtained from Ultra Scientific (North Kingston, Ontario, Canada) and was first dissolved in alcohol to achieve a stock solution of 10^{-2} M. Subsequent dilutions were performed with RPMI 1640 medium. The final concentration of alcohol did not exceed 0.5%. Equivalent volumes of alcohol were added to cells (vehicle controls). Aroclors 1016, 1221, 1248, 1254, 1260, 1262 and 1268 (Ultra Scientific) were dissolved at a concentration of 2000 ppm in RPMI 1640 medium supplemented with dimethyl sulfoxide (10% DMSO) (Sigma Chemicals). Subsequent dilutions were performed with RPMI 1640 medium. The final concentration of DMSO did not exceed 0.025%. Equivalent volumes of DMSO were added to cells (vehicle controls).

The thymus and lymph node were minced with forceps into complete RPM 1640 medium. Suspensions were filtered through gauze and then through nylon wool into a Pasteur pipette and washed once with complete RPMI 1640 medium. Blood was diluted in an equal volume of complete RPMI 1640 medium. Cells (thymocytes, lymph node cells or peripheral mononuclear cells (PBMCs)) were isolated by density gradient centrifugation using Ficoll Paque (Pharmacia Biotech Inc., Montreal, QC, Canada) for 35 min at 900 × g. Cells were then washed three times and suspended in complete RPMI 1640 medium. The cells were counted and their viability assessed using acridine orange-propidium iodide (Sigma Chemicals) with a fluorescence microscope.

PBMCs adjusted to a concentration of 2.63×10^6 cells/ml (95 µl) were plated with 95 µl of concanavalin A (Con A), to which was added either 10 µl of vehicle control (distilled water, alcohol or DMSO) or 10 µl of the contaminant to be tested (final concentration ranging from 10^{-9} M to 10^{-3} M for heavy metals and from 1.57 to 100 ppm for Aroclor mixtures). Cells were incubated 48h at 37℃ and 5% CO2 in 96-well tissue

culture plates. The optimal mitogen concentration used to stimulate T lymphocytes was established at 5µg/ml Con A (Lalancette et al., 2003). Each assay was plated in triplicate. After a 48-h incubation, 0.5 µCi (20 µl) of [^{3}H]-thymidine (6.7 Ci/mmol; ICN, Mississauga, Ontario, Canada) was added to each well and plates were incubated for a further 18 h. DNA was collected on fiberglass filters with a Titerteck cell harvester and the amount of radioactivity incorporated was evaluated with a Beckman β-scintillation counter. Raw data were expressed in disintegrations per minute (DPM). Results were presented as the percentage of the vehicle control. Only T lymphocytes were analyzed, because B lymphocytes are absent in the thymus.

For each analysis, the mean and standard deviation were determined on replicates for different chemicals. Differences between controls and treated groups were evaluated by one-way ANOVA followed by Dunnett's two-sided post-hoc test. The calculations were performed using STATISTICA for windows (version 7.0, StatSoft Inc, 1995). The error α was fixed at $P < 0.05$ for all tests.

Dose values for inhibition of 50% of the proliferation response (IC_{50}) (Table 1) indicate that no values for lymphocytes from blood exposed to Pb acetate, Aroclors 1016, 1221, 1248, 1254, 1260, 1262 and 1268 could be calculated because the proliferation response did not decrease below 50% for the range of tested concentrations. The same observation applied to thymocytes exposed to Aroclor 1221 and lymph node lymphocytes exposed to Aroclor 1221, 1254, 1260, 1262 and 1268.

RESULTS AND DISCUSSION

T lymphocyte proliferations varied between the different immune organs or tissues used as their source in this study. After exposure to heavy metals reductions of the proliferative response were similar for both lymphocytes from blood and lymph node. However, the IC_{50} value for thymocytes was lower than that for lymphocytes from blood and the lymph node (Table 1), suggesting that thymocytes were more sensitive to adverse effects of PCBs. Our result could indicate a higher sensitivity to PCBs exposure in thymocytes, which may indicate a higher susceptibility of immature thymocytes. The proliferation of T lymphocytes stimulated by Con A was significantly higher ($p < 0.05$) in blood than in both thymus and lymph node. However, Brousseau et al. (2003) demonstrated that in beluga, the stimulation index for Con A was higher for thymocytes than for peripheral blood mononuclear cells (PBMC). Because, in our study, preliminary assays could not be performed for thymocytes and lymph node cells, Con A

concentration used (5 µg/ml) was probably a suboptimal concentration. However, previous studies demonstrated that for PBMC from harbour and grey seals, 5 µg/ml was an optimal concentration (De Swart et al., 1995; Lalancette et al., 2003).

Metal	Organs, tissue	IC_{50}	PCB mixture	Organs, tissue	IC_{50}
MeHg	Blood	3.16×10^{-6} M	Aroclor 1016	Thymus	87.5 ppm
	Lymph node	2.18×10^{-5} M		Lymph node	77 ppm
			Aroclor 1248	Thymus	78.1 ppm
$ZnCl_2$	Blood	5.97×10^{-5} M		Lymph node	56.7 ppm
	Lymph node	6.00×10^{-4} M	Aroclor 1254	Thymus	64.6 ppm
			Aroclor 1260	Thymus	60.1 ppm
$CdCl_2$	Blood	1.78×10^{-5} M	Aroclor 1262	Thymus	70.3 ppm
	Lymph node	2.98×10^{-5} M	Aroclor 1268	Thymus	79.7 ppm
Pb acetate	Lymph node	5.67×10^{-4} M			

Table 1. Dose values for inhibition of 50% of the proliferative response (IC_{50}) to heavy metals and Aroclor mixtures in a grey seal.

Mercury (Hg) is a unique element that, unlike many metals, has no essential biological function. Toxic effects of Hg are well known but vary according to form. In general, ingested inorganic Hg tends to be poorly absorbed and distributed throughout the body, and as a consequence is less toxic than organic Hg (i.e. methylmercury). In our experiment, proliferation of grey seal lymphocytes - from blood and lymph node - was significantly reduced by 10^{-5} M CH_3HgCl. Lalancette et al. (2003) also found that lymphoblastic transformation of lymphocyte from blood in developing grey seals was reduced at a concentration of 10^{-5} M of CH_3HgCl. However, in harbour seal, lymphocyte proliferation was decreased at 10^{-6} M of CH_3HgCl (Das et al., 2008). Mercury is found mainly (up to 90%) in methylated form in the blood of marine mammals (Woshner et al., 2008) and the mean total Hg concentration reported in the blood of harbour seals was 172 µg/L (around 10^{-6} M) (Table 2) which is one order of magnitude lower than those of concentrations found to alter proliferation of lymphocytes in the present study. Thus, the

environmental exposure to Hg is too low to impair the health of grey seals but can affect the health of harbour seals.

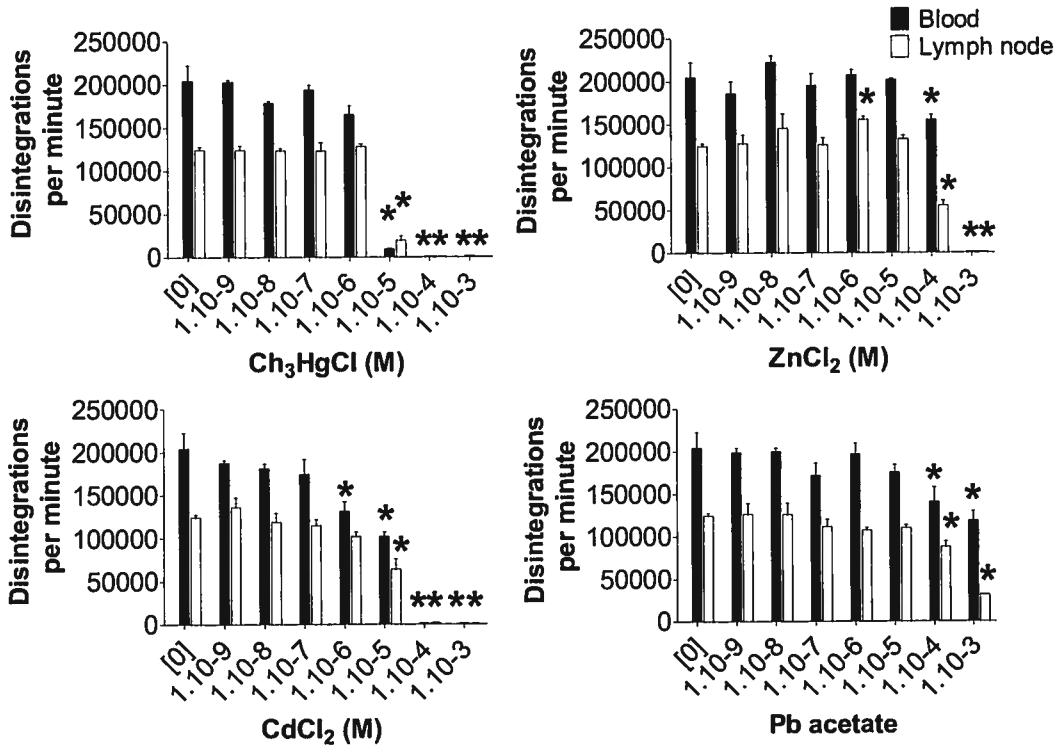

Figure 1 : Proliferation assay of grey seal T lymphocytes - from the thymus, lymph node and blood - following a 48-h exposure to four metals (triplicate assay). Lymphoblastic transformation stimulated by Con-A is measured as radioactivity emitted by [^{3}H]-thymidine incorporated in cells in DPM. Results are presented as disintegration per minute and given as mean ± standard error. One way ANOVA followed by Dunnett's two-sided post hoc test was performed to detect statistically significant differences between exposed groups and vehicle controls (* $p < 0.05$).

Zinc (Zn) plays a central role in mammalian physiology but little is known about the significance of different levels of zinc in marine mammals tissues (Das et al., 2003). Zn is essential in the development and function of the immune system in both humans and rodents, and is likely so in phocids. Proliferation of lymphocytes from blood and the lymph node was inhibited by exposure to 10^{-4} M $ZnCl_2$. In human and murine lymphocytes, DNA synthesis decreased at 5×10^{-4} M of Zn ions (Berger and Skinner, 1974) and 10^{-4} M of $ZnCl_2$ (Lawrence, 1981), respectively. Blood concentration of Zn (3150 µg/L = 4.82×10^{-5} M) reported in grey seals (Table 2), were close to the range of those affecting lymphocyte proliferation in the present study. If grey seal lymphocytes

behave *in vivo* as they do *in vitro*, the environmental exposure to Zn may result in concentrations bioavailable to blood and lymph node lymphocytes possibly high enough to eventually affect their proliferation. An immune suppression may result in increased susceptibility, incidence and severity of infectious diseases.

Cadmium (Cd) is regarded as one of the most toxic heavy metals. However, few studies on its toxic effects have been conducted in marine mammals, despite high levels encountered in several species (Das et al., 2003). In the present study, a significant inhibition of the proliferative response was noted at 10^{-6} M and 10^{-5} M $CdCl_2$ in blood and lymph node lymphocytes, respectively. Proliferation of thymocytes and splenocytes from beluga exposed to 10^{-5} M $CdCl_2$, was significantly reduced when compared to unexposed control cells (De Guise et al., 1996). The maximum concentration of total Cd reported in the blood of a grey seal (0.28 µg/L = 2.49×10^{-9} M) (Table 2) was three orders of magnitude lower than those of $CdCl_2$ found to alter proliferation of lymphocytes from blood in the present study. Thus, the environmental exposure to Cd is too low to impair the health of grey seals.

Although the main target for lead (Pb) toxicity appears to be the nervous system, immunotoxic effects have also been reported. McCabe and Lawrence (1991) indicated that Pb is not profoundly cytotoxic for most immune cells; yet it can cause major functional shifts within the immune system, as well as decreased host resistance to disease. Exposure to the highest concentrations (10^{-4} and 10^{-3} M) of $Pb(C_2H_3O_2)_2$ decreased lymphoblastic proliferation of lymphocytes from blood and lymph node. However, De Guise et al. (1996) reported that proliferation of beluga splenocytes and thymocytes was not affected by 10^{-4} M of lead chloride ($PbCl_2$). Alterations in mitogenic responses occurred in rat splenocytes at high concentrations (from 10-5 M to 10-3 M) of lead nitrate ($Pb(NO_3)_2$) (Lang et al., 1993). The maximum concentration of total Pb reported in the blood of a grey seal (0.13 µg/L = 6.27×10^{-10} M) (Table 2) was six orders of magnitude lower than those of $Pb(C_2H_3O_2)_2$ found to alter the proliferation of blood lymphocytes in the present study. Thus, the environmental exposure to Pb is too low to impair the health of grey seals.

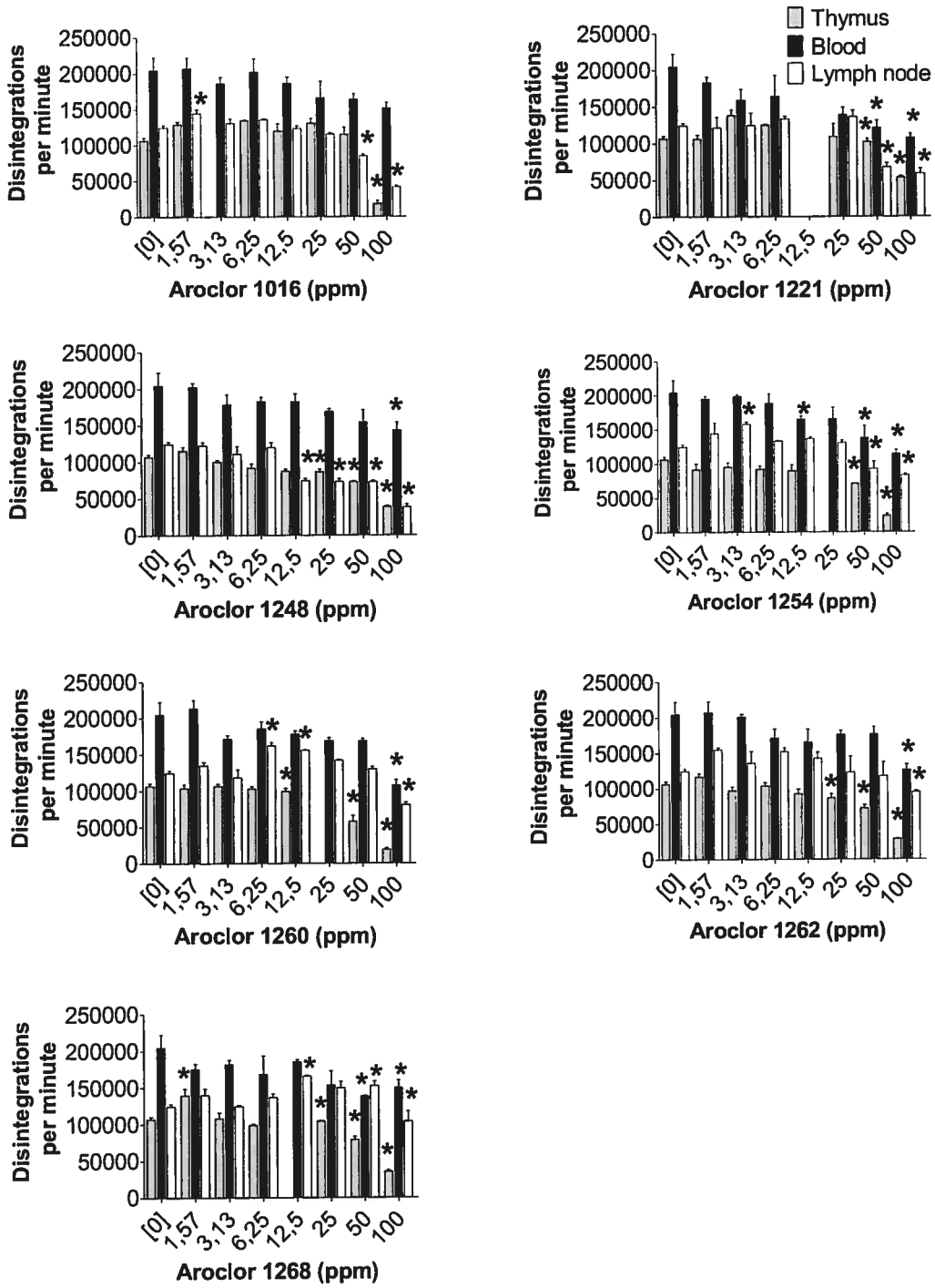

Figure 2 : Proliferation assay of grey seal T lymphocytes - from the thymus, lymph node and blood - following a 48-h exposure to seven Aroclor mixtures (triplicate assay). Lymphoblastic transformation stimulated by Con-A is measured as radioactivity emitted by [^{3}H]-thymidine incorporated in cells in DPM. One way ANOVA followed by Dunnett's two-sided post hoc test was performed to detect statistically significant differences between exposed groups and vehicle controls (* $p < 0.05$).

Polychlorinated biphenyls (PCBs) are highly stable, complex chemicals, most often found in mixtures called Aroclors that due to improper disposal practices have resulted in their wide distribution throughout the environment (Safe, 1994). Seven commercial PCB mixtures, Aroclors 1016, 1221, 1248, 1254, 1260, 1262 and 1268, significantly decreased T lymphocyte proliferation from 50 ppm, 50 ppm, 12.5 ppm, 50 ppm, 12.5 ppm, 25 ppm and 25 ppm, respectively. However, a significant stimulation of proliferation of T lymphocytes from lymph node occurred after exposure to Aroclors 1016 (1.57 ppm), 1254 (3.13 ppm), 1260 (6.25 ppm) and 1268 (12.5 ppm) and from thymus after exposure to Aroclor 1268 (1.57 ppm). Levin et al. (2005) reported that PCBs might also increase the proliferative response of (suboptimally stimulated) lymphocytes in harbour seals. Therefore, immunotoxic compounds might both suppress and stimulate, but stimulation could reflect a response of only the most immunocompetent cells or the effects of hormesis at low contaminant concentrations (Keller et al., 2006; Stebbing, 1982). Whereas chemical-induced immune suppression may result in increased susceptibility, incidence and severity of infectious diseases, conversely, immune enhancement may result in the loss of regulation within the immune system and can lead to adverse outcomes including autoimmune disease, anergy, and cancer (Descotes et al., 2000). Lahvis et al. (1995) indicated that a reduced Con A-induced lymphocyte proliferation in bottlenose dolphins was correlated with increasing whole blood concentrations of tetrachlorinated to octachlorinated biphenyls. Moreover, in C57BL/6 mice, the more highly chlorinated Aroclors (1260, 1254, 1248) were more toxic than the lower-chlorinated Aroclors (1242 and 1016) (Davis and Safe, 1989). Tri- and tetrachlorinated biphenyls predominate in Aroclors 1016 and 1221, tetra- and penta-chlorinated biphenyls predominate in Aroclor 1248, penta- and hexachlorinated biphenyls predominate in Aroclor 1254, hexa- and heptachlorinated biphenyls predominate in Aroclor 1260 and 1262, and octachlorinated homologues were most abundant in Aroclor 1268. The calculated IC_{50} values (Table 1) for thymocytes exposed to PCB mixtures in vitro in terms of toxicity can be ranked as follows : Aroclor 1260 > 1254 > 1262 > 1248 > 1268 > 1016 > 1221. Our results seem to suggest that toxicity is not linked to chlorination. Aroclor mixtures induced an inhibition of lymphoblastic proliferation at concentrations (15-50 ppm) higher than those reported in the blood of pinnipeds (Table 2).

Elements	Blood concentration	Species, Localisation	Reference
Zn	3.4 ± 0.5 mg/L 2.6-6.2 mg/L (*n* = 85)	Harbour seal (*Phoca vitulina*) Wadden Sea	Griesel *et al.* (2008) Includes data from Grielel et al. (2006)
	3.13 ± 0.11 µg/g (*n* = 6)	Southern elephant seal (*Mirounga leonine*) Antarctica	Baraj et al. (2001)
	3.15 ± 0.04 mg/L (*n* = 1)	Grey seal (*Halichoerus grypus*) Wadden Sea	Kakuschke et al. (2006)
Cd	<0.12 – 3.1 µg/L (*n* = 28)	Harbour seal Wadden Sea	Griesel *et al.* (2008)
	0.28 ± 0.01 µg/L (*n* = 1)	Grey seal Wadden Sea	Kakuschke et al. (2006)
	3.8 ng/g (*n* = 6)	Southern elephant seal Antarctica	Baraj et al. (2001)
	<0.005 – 0.01 µg/L (*n* = 1)	Weddell seal (*Leptonychoted weddellii*) Antactica	Yamamoto et al. (1987)
Hg	172 ± 143 µg/L	Harbour seal Wadden Sea	Das et al. (2008)
Pb	0.13 ± 0.03 µg/L	Grey seal Wadden Sea	Kakuschke et al. (2006)
	0.73 µg/L <0.02 – 4.52 µg/L *n* = 28	Harbour seal Wadden Sea	Griesel *et al.* (2008)
PCBs	201 – 1447 ng/g lipid *n* = 7	Harp seal (*Phoca groenlandica*) Greenland	Lydersen et al. (2002)
	50 ± 10 ng/g *n* = 14	Harbour seal San Francisco	Young et al. (1998)
	9.18 – 194 µg/L *n* = 47	Harbour seal North Sea	Weijs et al. (2009)

Table 2. Reported levels of total heavy metals and PCBs in the blood of pinnipeds.

In conclusion, these results demonstrate that, in one individual, the sensitivity of grey seal T lymphocytes to Hg and Zn at concentrations found in the blood of pinnipeds. However, concentrations of Cd, Pb and PCBs reported in the blood of pinnipeds were too low to impair lymphocyte proliferation. If grey seal lymphocytes from any individual behave *in vivo* as they do *in vitro* in this study then the ability of lymphocytes to proliferate might lead to the inhability to mount an adequate immune response in grey seals.

ACKNOWLEDGEMENTS

The authors want to express their gratitude to the Maurice-Lamontagne Institute for husbandry of the grey seal during the experiment. This work was supported by Fisheries and Oceans Canada through the St. Lawrence Vision 2000 fund and the US Environmental Protection Agency.

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4. DYNAMIQUE DES CONTAMINANTS

4.1. Dynamiques du transfert des contaminants de la mère à son jeune durant la lactation chez le phoque du Groenland, *Phoca groenlandica*

Article 4 : *Héloïse Frouin, Michel Lebeuf, Mike Hammill, Michel Fournier. Dynamics of contaminant transfer from mother to pup during lactation in harp seal Phoca groenlandica.*

Mon rôle a consisté à faire l'échantillonnage, les analyses, interpréter les données et écrire cet article à titre d'auteur principal. Cet article sera soumis à Chemosphere.

RÉSUMÉ

Sept couples mères-petits de phoque du Groenland (*Phoca groenlandica*) ont été échantillonnés durant la période de lactation près des îles de la Madeleine, QC, Canada. Des échantillons de gras et de sang des nouveau-nés, ainsi que des échantillons de lait, de gras et de sang de leurs mères ont été analysés pour déterminer les concentrations normalisées sur une base lipidique de PBDE, BPC et pesticides chlorés dans le but d'évaluer le transfert par la lactation de ces contaminants. Les concentrations normalisées sur une base lipidique dans ces tissus sont dans la gamme des ng g^{-1} lipide. Les concentrations de BPC dans le gras du phoque du Groenland démontrent une diminution temporelle, tandis que les concentrations de ∑DDT sont stables. Les transferts de PBDE, BPC et ∑DDT du gras maternel au gras du jeune sont de l'ordre de 51, 23 et 46%, respectivement. Les transferts de pesticides, de congénères de PBDE et de BPC diminuent avec les valeurs du $\log K_{ow}$ des analytes measures, les composés lipophiles étant plus facilement transférés aux jeunes par la voie de la lactation. Durant la lactation, le lait devient plus chargé en POPs (∑PBDEs, ∑PCBs, HCB and ∑DDT), de même que la charge en contaminants augmente dans la couche de gras du jeune. Ce transfert des POPs entre génération démontre l'importance relative de la lactation comme une voie d'élimination chez les femelles matures, et en parallèle une voie d'exposition chez les phoques nouveau-nés.

ABSTRACT

Seven mother-pup pairs of harp seal (*Phoca groenlandica*) were sampled during the lactation season among a breeding population of seals near Madgalen Islands, QC, Canada. The blubber and blood of the pups and the milk, blubber and blood of their mothers were analyzed for lipid-normalized concentrations of persistent organic pollutants (POPs) including polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) in order to evaluate their lactational transfer. The lipid normalized concentrations in these tissues were in the ng g^{-1} lipid range. PCB concentrations in blubber harp seals demonstrated a temporal decrease, whereas ∑DDT concentrations remained stable. Net transfer of ∑PBDEs, ∑PCBs and ∑DDT from maternal blubber to pup blubber was of order of 51, 23 and 46%, respectively. Lactational transfer of OCPs, PBDEs and PCBs decreased with the $\log K_{ow}$ (octanol/water partition coefficient) values of the analytes measured, lipophilic compounds being more readily transferred to the pups by the lactational route. Over the course of lactation, there was an increase in exposure to POPs (∑PBDEs, ∑PCBs, HCB and ∑DDT) as well as in contaminant burden in pup's blubber. At the same time, the contaminant burden in maternal blubber declined. This efficient inter-generational transfer of POPs demonstrates the importance of lactation as an excretory route in reproductive female seals and, in parallel, as exposure route in suckling pup seals.

Key words: Harp seals; pollutants; lactational transfer; *Phoca groenlandica*; mother-pup pair

INTRODUCTION

Marine mammals inhabiting the Gulf of St. Lawrence are exposed and bioaccumulate substantial amounts of persistent organic pollutants (POPs) (Beck et al. 1994; Hobbs et al. 2002; Gouteux et al. 2005; Frouin et al. 2009b). One of the marine mammals inhabiting the Gulf includes Northwest Atlantic harp seals (*Phoca groenlandica*). These animals overwinter in the St. Lawrence Marine Ecosystem (SLME), but summer in Arctic waters (Stenson and Sjare 1997).

In the first half of March, female harp seals give birth on the drifting pack ice in the Gulf and pupping takes place during a 2-3 week period, and the entire breeding season usually lasts less than six weeks (Stewart and Lavigne 1980). Lactation lasts for only about 12 days (Sergeant 1973; Kovacs and Lavigne 1985; Kovacs 1987) during

which time the pups more than triple in weight (Lydersen and Kovacs 1999). During the nursing period, pups grow at a rate in excess of 2.0 kg/day (Stewart and Lavigne 1980; Kovacs and Lavigne 1985) on the fat-rich milk their mothers provide (Van Horn and Baker 1971; Lavigne et al. 1982). Like most phocids, females do not normally ingest significant quantities of food during lactation (Seargent 1973; Nilssen et al. 1995). Stewart and Murie (1986) found that approximately one per cent of female harp seals had more than trivial amounts of food in their stomachs during the nursing season.

Lactation is the most energetically costly component of mammalian reproduction and represents a significant portion of the energy budget of an adult female mammal (Millar 1977). Suckling is the major route of exposure to high concentrations of lipophilic contaminants for mammalian neonates, as well as the major route of elimination of these chemicals for the dams through lactation (Addison and Brodie 1987). In marine mammals the process may be particularly important, since relatively large amounts of depot lipid are mobilised for production of a lipid-rich milk, usually over a short period (King 1983). During gestation, lipophilic chemicals easily traverse placental membranes and are therefore transferred in variable quantities to the foetus (Tanabe et al. 1982). During lactation there is further mobilization of lipophilic chemicals from the blubber to the milk, and the transfer of these contaminants to offspring continues (Wolkers et al. 2002). Other than reproductive transfer in females, contaminant loads in mammals can be reduced through the biotransformation and subsequent excretion of organochlorine and organobromine metabolites in the bile, urine and feces.

Many studies have addressed the mechanisms involved in POPs transfer during lactation between phocid mothers and pups (Addison and Brodie 1987; Sørmo et al. 2003; Wolkers et al. 2004). These studies have shown that the transfer of different POPs from maternal lipid stores to pups appears to occur selectively, implying that some POPs are retained in the mother. Moreover, recent studies have shown that seal milk contains substantially more contaminants than a "normal" seal diet of fish and crustaceans (Wolkers et al. 2002). In fact, seal pups feed one trophic level higher than their mothers and are concomitantly exposed to relatively higher contaminant concentrations. Previously, Wolkers et al. (2002) studied the concentrations of PCBs and OCPs in blubber and milk of mother and pup harp seals from SLME. However, in pinnipeds, knowledge on the transfer of some POPs, notably PBDEs, and how they are partitioned in maternal and offspring tissues during the course of lactation is poorly understood. The aim of this study was to examine the qualitative and quantitative partitioning of

chlorinated POPs (pesticides and PCBs) and brominated POPs (PBDEs) in maternal blubber, blood and milk, and blubber and blood pup, of harp seals from early to late in the lactation period. The present study also examines the transfer efficiencies of these contaminants from the mothers to the nursing pups.

MATERIAL AND METHODS

Sample collection and storage

The study was conducted near the Magdalen Islands in the Gulf of St Lawrence in March 2008. Six mother-pup pairs were captured twice between birth and weaning to collect milk, blubber (mothers and pups) and blood (mothers and pups) at different lactation stages (details in Table 1). One mother-pup pair was captured only one time throughout lactation. Prior to sampling, animals were weighed (to ±0.5 kg), tagged, and their sex noted. Each pup was aged from appearance and from its body weight and the relationship, age (days) = 0.35*body weight (kg)-3.2, developed for the same population by Stewart and Lavigne (1980).

In pups, a biopsy was taken through the skin into the subcutaneous blubber layer using a 6 mm biopsy punch. In mothers, an attempt was made to obtain a full blubber depth. The site was prepared with an antiseptic scrub and a local anesthetic was injected. A modified drill was used to extract the biopsy. Samples were wrapped in solvent rinsed aluminium foil, placed in a sealed plastic jar, were kept in a cooler with ice packs for up to 10 h in the field and then stored at -20℃ until analysis. Blood samples were taken from the extradural intravertebral vein into Vacutainer tubes (Becton-Dickinson, New Jersey, USA), were kept in the field at 4℃ until processing (within 10 h). Blood samples were centrifuged and the sera were frozen at -20℃ until analysis. Standard length and axillary girth were measured; the seal was positioned on its ventrum with its head and vertebral column in a straight line. Body condition was assessed by calculating a condition factor for each seal (axillary girth/standard length, multiplied by 100; (McLaren 1958)). All research was approved by the Animal Care Committees of Fisheries and Oceans Canada.

Chemical analysis of POP

The methodology for extraction, cleanup and quantification of target analytes (PBDEs, PCBs and OCP) in blubber by gas chromatography (GC)/mass spectrometry

(MS)/mass spectrometry, as well as QA/QC procedures are reported elsewhere (Hobbs et al. 2002; Lebeuf et al. 2004), and is briefly presented below. Between 0.06 and 0.27 g of blubber was dried with ~ 20 g anhydrous Na_2SO_4, spiked with surrogates $^{13}C_{12}$ – labelled PCB congeners, -labelled PBDE congeners and pesticides. A fraction (10%) of the extract was used to lipid determination. The rest of the extract was cleaned and eluted then cleaned extract was concentrated and was analyzed by GC/MS/MS.

Seal milk samples were analyzed for the target compounds by homogenizing about 5 g of each sample with sodium sulphate. The dried samples were packed into glass chromatography columns and spiked with a surrogate solution containing a mixture of $^{13}C_{12}$–labelled PCB congeners, -labelled PBDE congeners and pesticides. A fraction (10%) of the extract was used to lipid determination. The extracts were further cleaned-up, eluted and then cleaned extract was concentrated and target compounds were quantified by GC/MS/MS.

Seal serum samples received a mixture of $^{13}C_{12}$ internal standards extracted with hexane:methyl tert-butyl ether. After evaporation of the elution solvent the recovery standard was added and the final volume was reduced to 100 µl. The obtained extract was analyzed by GC/MS/MS.

Seal blubber, milk and serum were analyzed for 39 PBDE congeners, from di to hepta-BDE homolog groups, 43 PCB congeners, from di to deca-PCB homolog groups and 16 OCPs (4,4,'-DDE, 4,4'-DDD, 2,4'- and 4,4'-DDT, dieldrine, α- and γ-chlordane, heptachlor, oxychlordane, cis- and trans-nonachlor, α-, β- and γ-hexachlorocyclohexane (HCH), hexachlorobenzene (HCB) and mirex). In blubber, the limits of quantification of PBDEs, PCBs and pesticides were 2.42 ng g-1 lipid, 7.12 ng g^{-1} lipid and 3.84 ng g^{-1} lipid, respectively. The limit of quantification in serum was established at 15.81 ng g^{-1} lipid for PBDEs, at 13.69 ng g^{-1} lipid for PCBs and at 22.96 ng g^{-1} lipid for OCPs. Quantification limits for PBDEs, PCBs and OCPs in milk were 0.80 ng g^{-1} lipid, 0.01 ng g^{-1} lipid and 12.33 ng g^{-1} lipid, respectively.

Lipid determination

Lipid contents in blubber and milk were determined gravimetrically by measuring the total non-volatile solvent extractable material.

Percent lipid was calculated using the following equation: % lipid = (mass of lipid/mass of sample) x 100%.

Blood lipids were measured using the colorimetric technique of Frings et al. (1972). Briefly, using 100 µl serum from each seal sample, lipids were hydrolyzed with sulphuric acid and then reacted with phosphor-vanillin, producing a chromophore (absorption maximum 540 nm). Sample absorption was determined using ultraviolet/visible (UV/Vis) spectroscopy (Thermo Electron Instruments MULTISKAN ASCENT), and lipid concentration was quantified using a linear, five-point calibration curve of olive oil standards that bracketed all analyzed samples.

QA/QC

One procedural blank and one Standard Reference Material (SRM 1945) were included with every 10-blubber sample analysis for PBDE concentrations and lipid determinations. A pilot whale (*Globicephala sp.*) blubber sample available as the standard reference material SRM 1945 was obtained from the National Institute of Standards and Technology (NIST). Repeated analysis of SRM 1945 (n = 5) indicating that the precision of the method expressed by coefficients of variation (CV) for all detected PBDEs varied between 9 and 20% of the average values. CV for all detected BPCs varied between 1 and 30% of the average values. OCPs were within 27% of the expected values.

The milk samples were analysed in batches of 12, which included one blank, one cow milk sample, one cow milk fortified sample and nine samples. Repeated analysis of cow milk fortified (n = 2) indicated that the precision of the method expressed by CV for all detected PBDEs, PCBS and OCPs varied between from 1 to 21%, from 2 to 24% and from 2 to 18%, respectively.

The blood samples were analysed in batches of 12, which included one blank, one fortified blank, one human serum sample available as the standard reference material SRM 1589a (NIST) and nine samples. For all detected PBDEs, the CV of replicate analysis of SRM 1589a (n = 5) and blank fortified (n = 5) were respectively within 22% and 9 % of the average values. For all detected PCBs, the CV of replicate analysis of SRM 1589a (n = 5) and blank fortified (n = 5) were respectively within 28% and 24 % of the average values. For all detected PCBs, the CV of replicate analysis of SRM 1589a (n = 5) and fortified blank (n = 5) were respectively within 16% and 18% of the average values.

The average recovery of the labelled surrogates was 99 ± 13 %.

Table 1. Size, lipid content, and day of development of mother-pup pairs harp seals sampled near Madgalen Islands at two sampling times.

Sample n°.	Seal code	Length (cm)	Weight (kg)	Girth (cm)	Age (estimated in days) and sex of pup	% Lipid content		
						Blubber	Milk	Blood
1st sampling								
# 1 Mother	797	157	134	nd	Nd, F	81.5	51.8	0.9
# 1 Pup	798	75	16.5	64	3, F	71.3		1.3
# 2 Mother	696	152	135	nd	nd	81.5	55.4	0.9
# 2 Pup	694	89	23.5	74	6, F	82.1		1.9
# 3 Mother	691	142	112.5	nd	nd, F	72.9	53.2	0.9
# 3 Pup	692	78	19.5	70	4, M	65.5		1.7
# 4 Mother	699	147	104	nd	nd, F	83.0	56.5	0.9
# 4 Pup	700	81	16	65	3, F	62.3		1.1
# 5 Mother	665	159	130.5	nd	nd, F	73.3	45.1	0.9
# 5 Pup	664	86	15	71	2, M	74.3		1.3
# 6 Mother	666	155	139.5	nd	nd, F	81.2	24.8	0.9
# 6 Pup	667	80	17.5	72	3, F	84.5		1.4
# 7 Mother	662	154	152.5	nd	nd, F	69.9	45.2	1.0
# 7 Pup	661	72	8.5	54	1, M	72.5		1.5
2nd sampling								
# 1 Mother	797		121	nd	nd, F	79.9	53.7	0.8
# 1 Pup	798	84	26	74	7, F	88.1		1.3
# 2 Mother	696		118	nd	nd, F	83.1	55.6	0.8
# 2 Pup	694	89	33	90	10, F	79.4		1.6
# 3 Mother	691		99	nd	nd, F	71.9	48.1	0.7
# 3 Pup	692	89	28	78	8, M	86.7		1.1
# 4 Mother	699		95	nd	nd, F	76.2	43.3	0.8
# 4 Pup	700	89	26	77	7, F	81.2		1.5
# 5 Mother	665		121	nd	nd, F	80.7	52.2	0.9
# 5 Pup	664	88	20	73	5, M	83.9		1.3
# 6 Mother	666		130	nd	nd, F	89.0	37.7	0.7
# 6 Pup	667	83	24.5	77	6, F	91.6		1.2

Data analysis

Concentrations of total ∑PBDEs and all congeners reported individually are in nanograms of analyte per gram of sample and lipid normalized for intercomparison. The differences in maternal blubber, blood, and milk and pup blubber and blood POP concentrations were investigated using a single factor analysis of variance (ANOVA) followed by a Tukey's post-hoc comparisons test.

Transfer ratio for each compounds measured was calculated by dividing the compound concentration in the milk by the concentration in the maternal blubber (transfer from mother into the milk) or the compound concentration into the blubber pup by the concentration in the maternal blubber (transfer from mother to pup). Relationship between transfer ratio values and $\log K_{ow}$ (octanol/water partition coefficient) for PBDEs, PCBs and OCPs were performed using the linear regression model.

The significance level adopted was $P < 0.05$. Statistical analyses were performed using the software Statistica for windows (version 7.0, StatSoft Inc, 1995).

Changes throughout the lactation

The model tracks the accumulation and losses of contaminants in a mother-pup pair on a daily basis from parturition (day 0) until weaning (12 days). The body was modelled as two compartments representing the sculp (skin with subcutaneous blubber attached) and the milk.

The sculp weight of harp seal pups and nursing females were calculated based on the relationship from Stewart and Lavigne (1984):

(1) Pup sculp weight (kg) = 2.12 +1.95×pup age

(2) Mother sculp weight (kg) = 63.01-2.63×pup age

combinated with:

(3) Pup body weight (kg) = 9.3+2.3×pup age (Oftedal et al. 1989)

(4) Mother body weight (kg) = 135.4 -3.1×pup age (Kovacs et al. 1991)

The sculp to body weight (%) could then be calculated for each animal using a polynomial equation based on combination of equations of sculp weight and body weight:

(5) Sculp to body weight for pup (%) = 26.61+7.9×pup age-0.38×pup age^2

(6) Sculp of nursing female weight (%) = 46.48-0.81×pup age-0.03×pup age^2

The daily sculp burden was calculated as:

(7) daily sculp burden in pup (µg) = pup weight (kg) × % sculp of pup weight × % lipid content × contaminant concentration (µg kg^{-1})

(8) daily sculp burden in nursing female (µg) = mother weight (kg) × % sculp of nursing female weight × % lipid content × contaminant concentration (µg kg^{-1})

Nursing female harp seals fast during lactation and lose a fraction of their fat to produce milk. This transfer fat is associated with a transfer of liposoluble chemicals.
The average daily milk consumption for pups was 3.65 ± 0.24 kg (Lydersen and Kovacs 1996). The daily exposure (daily milk contaminant intake) was then calculated using the average daily milk consumption of pups and the percentage lipid of milk:
(9) Daily exposure (µg) = % of lipids in milk × 3.65 (daily milk consumption kg) × contaminant concentration (µg kg^{-1})
Values of « daily sculp burden » and « daily exposure » were normalised on the basis of 5th day of lactation.

RESULTS AND DISCUSSION

3.1 Concentrations of POPs

In harp seal blubber and milk, the relative concentrations of the compounds measured followed the pattern: ∑PCB > ∑DDT > ∑CHLs > HCB > ∑HCH, ∑PBDE > Mirex (Table 2). However, our results differed from another study on harp seals demonstrating than in nursing females sampled in 1999 near the Madgalen Islands the relative concentrations of persistent organochlorines followed the pattern: ∑PCB > ∑CHLs > ∑DDT > ∑HCH > HCB (Wolkers et al. 2002). Moreover, in nursing female grey seals sampled in 1995 in Gulf of St. Lawrence, the concentrations of POPs followed the pattern: ∑PCB > ∑CHLs > ∑DDT > ∑HCH > HCB > Mirex (Sørmo et al. 2003).

Overall, the tetra-, penta- and hexa-brominated PBDEs were the most prevalent PBDEs, the penta-, hexa- and hepta-chlorinated PCBs were the most prevalent PCBs, while ∑DDT was the most abundant pesticide (Table 2).

The use of PCBs was discontinued in the 1970s. Compared to concentrations in blubber of female (391 ng g^{-1} lipid ng/g lipid weight basis) and pup (148 ng g^{-1} lipid ng/g lipid weight basis) harp seals sampled in 1999 in the same area (Wolkers et al. 2002), the concentrations in female (754.4 ± 171.9 ng g^{-1} lipid) and pup (160.2 ± 63.5 ng g-1 lipid) harp seals from the present study showed 2 and 3 times lower blubber concentrations of PCBs, respectively.

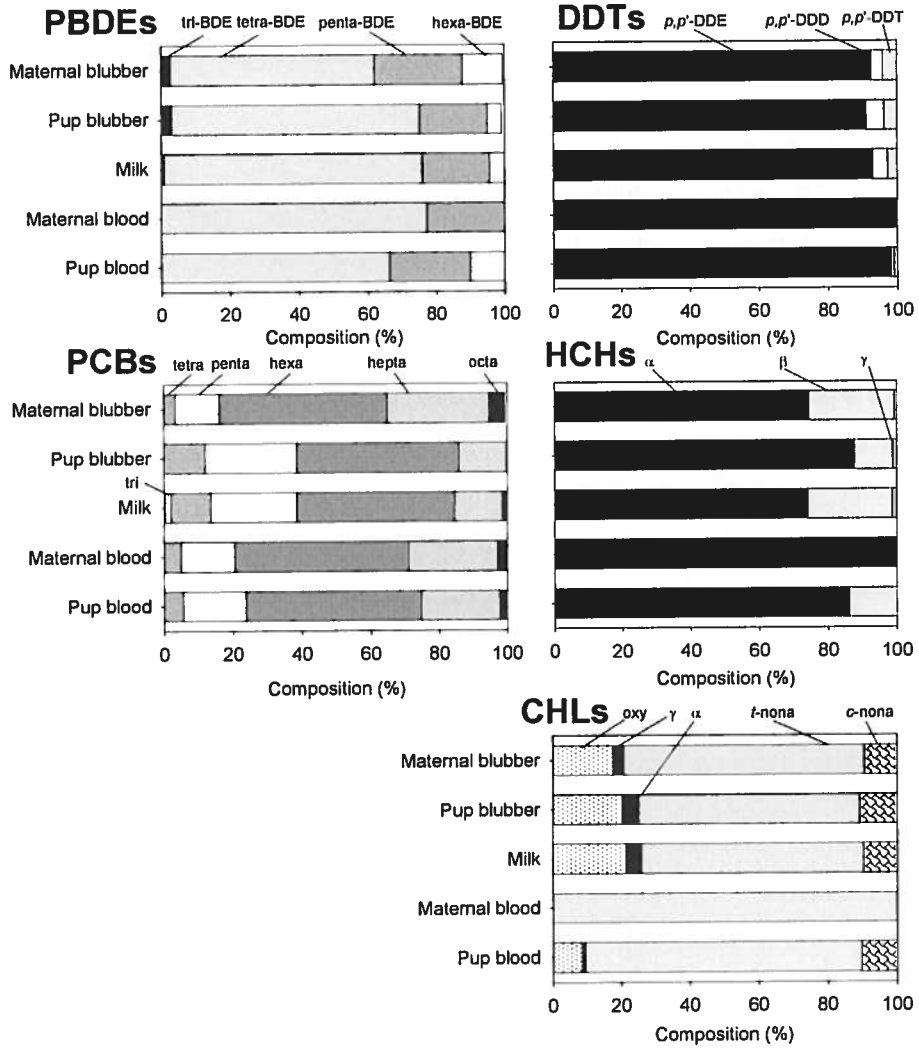

Figure 1. Composition (%) of PBDE congeners, PCB congeners, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane (DDT) compounds, hexachlorocyclohexane (HCH) compounds and chlordane (CHL) compounds in blood, blubber and milk tissues of harp seals. Data from early/mid and late lactation were combined.

Although it was banned in the United States and Canada in the early 1970s, DDT-related substances can still be detected in air from southern Mexico, Belize, eastern USA and western and eastern Canada (Shen et al. 2005). The fraction of ∑DDT represented by p,p'-DDE - an indication of the "age" of DDT group (Aguilar 1984) – was significant lower in 1999 (about 80%) (Wolkers et al. 2002) that in the present study (about 93%). This suggests that the availability of DDT in the Gulf and/or Arctic may be decreasing between 1999 and 2008 and that the biotransformation of DDT to DDE may be increasing, as previously suggested (Addison et al. 1984; Beck et al. 1994). The

decrease in ∑DDT is particularly clear. Compared to concentrations in blubber of female (1350 ng g^{-1} lipid weight basis) and pup (427 ng g^{-1} lipid weight basis) harp seals sampled in 1999 in the same area (Wolkers et al. 2002), the concentrations in female (312.2 ± 72.0 ng g^{-1} lipid) and pup (139.8 ± 43.0 ng g-1 lipid) harp seals from the present study showed 4 and 3 times lower blubber concentrations of ∑DDT, respectively.

Chlordane was widely used in the 1970s following bans in the United States, Canada and Europe on the extensive use of DDT. In the United States, regulatory bans on chlordane use in agriculture were imposed in 1983, due to concerns about carcinogenicity and environmental persistence and bioaccumulation. Chlordane use continued in many countries, including the United States, for non-agricultural purposes (e.g., termite control) until the mid-1990s. Compared to concentrations in blubber of female (722 ng g^{-1} lipid weight basis) and pup (254 ng g^{-1} lipid weight basis) harp seals sampled in 1999 in the same area (Wolkers et al. 2002), the concentrations in female (300.2 ± 82.3 ng g^{-1} lipid) and pup (134.8 ± 31.1 ng g-1 lipid) harp seals from the present study showed 2 times lower blubber concentrations of ∑CHL.

HCH has been used as a pesticide since 1942 when the insecticidal properties of the γ isomer were discovered. Technical HCH consists of an approximate composition of 60-70% α, 5-12% β, 10-15% γ, 6-10% δ, and 3-4% ε (Kutz et al. 1991). Nowadays lindane is still used in specific applications such as insect control in fruit, grain and vegetable crops in forestry, for vector control and seed treatment. Compared to concentrations in blubber of female (71.3 ng g^{-1} lipid weight basis) and pup (65.2 ng g^{-1} lipid weight basis) harp seals sampled in 1999 in the same area (Wolkers et al. 2002), the concentrations in female (29.3 ± 12.7 ng g^{-1} lipid) and pup (31.0 ± 18.4 ng g-1 lipid) harp seals from the present study showed 2 times lower blubber concentrations of HCH.

There are three types of commercial PBDE products including the penta-BDE mixture (DE-71 and Bromkal 70-5DE), the octa-BDE mixture (DE-79 and Bromkal 79-8DE) and the deca-BDE product (Saytex 102E and Bromkal 82-ODE). However, due to growing environmental and human health concerns, two of the three commercial formulations (Penta and Octa) were removed from the European (1998) and North American (2004) marketplace (Ross et al. 2009). Deca-BDE formulation was removed in Europe in 2008 but is poised for a ban in the national markets in Canada and the USA (some US states have regulated Deca-BDE) (Ross et al. 2009). The PBDEs were detectable in all tissues (excepted for the hexa-BDE in maternal serum), but in relatively low concentrations with tetra-and penta-BDE being most prevalent. In blubber of two

harp seals sampled in Greenland between 1999 and 2001, ∑PBDE concentration was 45.7 ± 54.7 (ng g^{-1} wet weight) (Johansen et al. 2004), which was similar to ∑PBDE concentration measured in maternal blubber of the present study (37.7 ± 16.1 ng g^{-1} lipid weight).

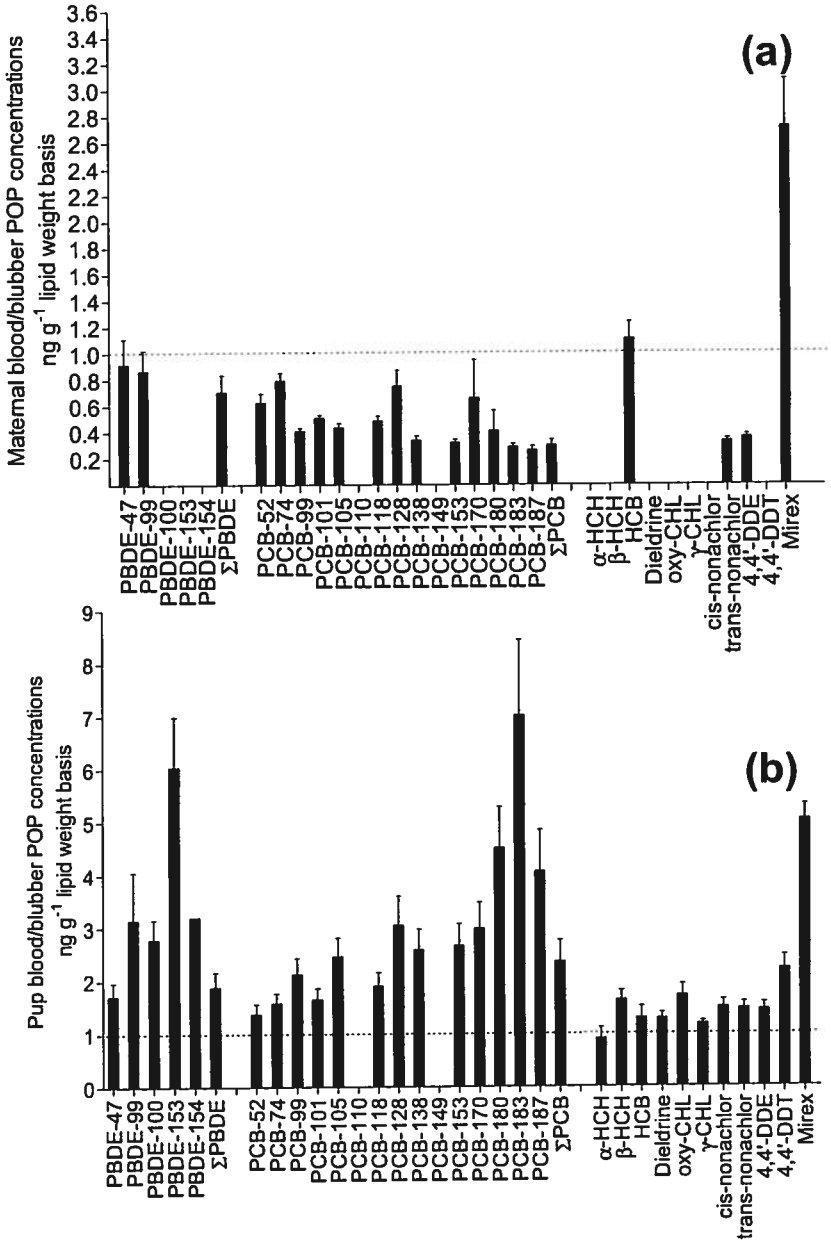

Figure 2. Maternal blood (a) and pup blood (b) POP concentrations relative to corresponding concentrations (ng g^{-1} lipid weight basis) in maternal blubber and pup blubber in harp seal mother-pup pairs. Data from early/mid and late lactation were combined.

Patterns of POPs in tissues and transfer-ratio

POPs in biological material are often expressed on a lipid basis as these compounds tend to accumulate in lipids. Partitioning of POPs between the different tissues and the efficiency of transfer between two tissues was assessed using lipid-normalized concentrations.

The contribution of penta-, hexa-, and hepta-PCB to ∑PCBs differed between females, milk and pups (Fig. 1). The pattern was similar between blubber pup and milk and maternal and pup serum. Penta- and hexa-PCB dominated in blubber pup (27% and 47%, respectively) and milk (25% and 46%, respectively) samples. In maternal blubber, hexa- and hepta-PCB dominated with 49% and 30%, respectively. Hexa- and hepta-PCB dominated in maternal serum (51% and 26%, respectively) and serum pup (51% and 23%, respectively) samples.

p,p'-DDE dominated in all samples (blubber, blood and milk) (Fig. 1). The pattern was similar between milk, blubber pup and maternal blubber but different with serum samples. The pattern can only be adequately compared when all constituents are detected. However, p,p'-DDT and p,p'-DDD were under quantification limit in maternal serum. This observation could bias the pattern. Among CHLs, trans-nonachlor was the major compound, followed by oxychlordane and cis-nonachlor (Fig. 1). Trans-nonachlor and α- and γ-chlordane are metabolised to the more stable epoxide, oxychlordane, a process which is thought to occur to some extent in organisms occupying low trophic positions and to a greater extent in organisms such as pinnipeds (Kawano et al. 1988). In harp seal samples from the Gulf of St. Lawrence, trans-nonachlor was the predominant compound amongst the chlordane analytes measured in maternal blubber, pup blubber, and in the milk, corresponding to 70%, 64% and 64% of the total chlordanes, respectively. Our results were higher than those from the study of Wolkers et al. (2002). The pattern of CHLs was similar between milk and maternal blubber and blubber pup samples (Fig. 1), and in serum pup samples, oxychlordane was less important. As previously mentioned, the pattern can only be adequately compared when all constituents are detected. So, the different pattern observed in maternal serum could be explain by the fact that only α-HCH was above the quantification limit. Concentrations of oxy-chlordane cis-nonachlor, α-chlordane and γ-chlordane were under the limit of quantification in the maternal serum samples (Fig. 2a).

The contribution of α-, β-, and γ-HCH, to total HCH differed between females, milk and pups. Lindane (γ-HCH) was a minor fraction of ∑HCH in seal blubber and milk.

Alpha-HCH dominated in all samples, making up about 74 % in the milk and the maternal blubber, about 86 % in pup blubber and serum, and 100% in maternal serum. The pattern of HCHs was similar between milk and maternal blubber and between blubber pup and serum pup samples (Fig. 1). As previously mentioned, the pattern can only be adequately compared when all constituents are detected. So, the different pattern observed in maternal serum could be explain by the fact that β-HCH and γ-HCH were below the quantification limits. Alpha-HCH was previously described as the major HCH isomers in several seal species, such as harp seals, ringed seals (Phoca hispida) and bearded seals (Eringnathus barbatus) (Muir et al. 2003). The compositions of DDT, HCH and CHL compounds in harp seal tissues are indicative of the lack of fresh inputs of these compounds in Gulf of St. Lawrence/Arctic environment. Moreover, a greater biotransformation capacity could also lead to the predominance of metabolites rather than the parent compounds in tissues.

Tetra- and penta-BDE dominated in all samples (blubber, blood and milk) (Fig. 1). The pattern of PBDEs was similar between milk and blubber pup and between maternal blubber and serum pup samples. As previously mentioned, the pattern can only be adequately compared when all constituents are detected. So, the different pattern observed in maternal serum could be explain by the fact that hexa-BDE was below the quantification limit.

Concentrations of several individual PCBs (PCB-110, -149) and PBDEs (BDE-100, -153 and -154) were under the limit of quantification in the serum samples (Fig. 2a) Except Mirex and HCB, the mean ratio of maternal serum/blubber was below the unity (value 1) (Fig. 2a). On the contrary, except α-HCH and γ-CHL, in pups the mean ratio was higher than unity, indicating enrichment of contaminants in serum versus blubber (Fig. 2b). These results indicate that the contaminant pattern varies from one tissue to the other. The POP patterns may result from the physicochemical properties of the different contaminants and of the lipid composition of the tissue considered in seal pups. The lipid composition in blood of young seals is mainly constituted of non-polar lipids (triglycerides and cholesterol esters) (Nelson 1970) in which highly brominated and chlorinated congeners, which are more lipophilic and less polar, may be more soluble (Apostoli et al. 2005) than in non-polar triacylglycerol blubber lipid during lactation period. Moreover, prodigious reserves of triacylglycerol accumulate in blubber of seal pups during the first few days of neonatal development; the high levels attained at this time

are maintained during subsequent development (Bailey et al. 1980). Thus, PBDEs and PCBs transferred by lactation could be, in a first time, linked to lipids of blood pup and slowly deposit in blubber pup throughout the lactation. The observed differences in contaminant concentrations and patterns between the tissues stress the fact that depending on which tissue is selected in a contaminant study or monitoring program, results obtained might be in a way "tissue specific". Among pups both blood and blubber represent accumulation of pollutants over a short time period while in adults, blood levels represent short term accumulation of pollutants whereas blubber integrates the accumulation of pollutants over longer durations. Wolkers et al. (2006) demonstrated that no correlation existed in ΣPBDEs concentrations between plasma and blubber, suggesting that plasma of adult and pup hooded seals overestimated ΣPBDEs. Moreover, Lydersen (2002) concluded that blood plasma was an unreliable indicator for contaminant exposure to PCBs and OCPs in adult harp seals.

The lipid-normalized concentration ratios of most compounds measured were significantly lower in samples of maternal milk and pup blubber than in those of maternal blubber (Fig. 3a,c). The relative lipid-normalized concentrations for almost all analytes measured followed the pattern: maternal blubber > milk > pup blubber. The differences are particularly apparent for some of the highly chlorinated PCB congeners, chlordanes, DDT isomers and mirex (Fig. 3a,c). This pattern of lower lipid-normalized concentration ratio of contaminants in milk relative to the maternal blubber is consistent with the literature on the levels of lipophilic contaminants in the milk of phocid seals (Bacon et al. 1992; Kleivane et al. 1997; Beckmen et al. 1999; Wolkers et al. 2002; Miranda Filho et al. 2009). Contaminant transfer from female to pup in seals through the milk favors the transfer of relatively more polar contaminants to the pups (Fig. 3c). Consequently, pups are exposed to a different contaminant cocktail than the adults (similar contaminants, but in slightly different concentrations and proportions).

The maternal milk/blubber ratios were low for all PCB and PBDE congeners (Fig. 3a). These observations were similar to maternal/pup blubber ratios (Fig. 3c). The milk/pup blubber concentration ratios were similar or higher to unity for all contaminants (Fig. 3b). Our ratios combinated early/mid and late of lactation, so the slightly lower concentrations of some POPs in the pup blubber compared to the milk, is probably because POP concentrations increasing in the milk throughout the lactation (Sørmo et al. 2003)and pup blubber is in formation. Since the pups are born with almost no subcutaneous blubber, so the samples collected early in lactation represent newly

deposited blubber synthetised from relatively "clean" milk. As lactation progresses pups deposit more blubber from the increasingly contaminated milk. However, this deposition occurs in the inner blubber layer of the pup, and does not affect the outer "older" layer (the inner blubber layer is thinner than the outer "older" layer in our samples).

The observed differences in contaminant pattern in females and their milk as well as in the relative pup concentrations indicate selective transfer from mother to pup. Contaminant lipophilicity, which can be expressed as the octanol-water partitioning coefficient, log $K_{o/w}$, showed a clear relation with both the relative pup blubber concentration or the milk concentration and the relative contaminant presence in female blubber (Fig. 4a and 4b). In fact, a significant inverse correlation was observed between the log $K_{o/w}$ values and the transfer rate from female to pup for all PCBs analyzed and pesticides. These findings are consistent with results from grey seals (*Halichoerus grypus*) (Addison et al. 1999), harp seals, hooded seals (*Cystophora cristata*) (Espeland et al. 1997) and southern elephant seals (*Mirounga leonina*) (Miranda Filho et al. 2009). Moreover, transfer efficiency of PBDEs from mother to pup during lactation declined consistently with increasing log $K_{o/w}$. This result was previously observed in grey seals (Ikonomou and Addison 2008). Since, transfer ratios are higher between milk/maternal blubber than between pup/maternal blubber, this indicates higher transfer rates between maternal blubber to milk than between maternal blubber to blubber pup. These observations demonstrated that the process of transfer may be further complicated by biological membranes, notably the mammary tissue.

Exposure and burden with time

It was estimated that about 98% of the contaminant residues in grey seal pups sampled at weaning came from maternal lactational transfer (Addison and Stobo 1993). Data from the present study show that there is significant accumulation of organochlorine and bromine residues in harp seal pups as a result of lactational transfer of these compounds from the mother. The short lactation period of harp seals is associated with a massive mobilization and transfer of lipophilic contaminants by blubber lipids from female to pup (Espeland et al. 1997). When comparing data involving pup mass gain to maternal mass loss among individual mother-pup pairs, harp seals rank highest among the phocids with a mass transfer rate of 77 % (Kovacs et al. 1991). However, the transfer rates of contaminants were lower. In our study, total PBDE transfer from mother to pup was 50.5 ± 22.9%, this is similar to total PBDE transfer (44.4 ± 9,9%) in mother-

pup pair grey seals (Ikonomou and Addison 2008). In the present study, this transfer was more than the transfer of PCBs which was estimated to be 22.7 ± 11.3% and similar to ∑DDT (46.1 ± 14.4%). To best understand the evolution of contaminant transfer throughout lactation period, daily exposure (intake by daily milk consumption) and daily sculp burden in mothers and pups were calculated (cf. equations in material and methods).

Daily exposure to ∑PBDEs, ∑PCBs and ∑DDTs increased significantly during the lactation period, daily exposure increased but not significantly with HCB (Fig. 5). Although in our study, increase of lipid content in milk throughout lactation was less obvious than in Debier et al. (1999), the daily exposure of PBDEs, PCBs and HCB received by pups increased during lactation period as being affected by increasing lipid content in milk.

Daily sculp burden decreased significantly with HCB and not significantly with ∑PCBs and ∑DDTs in mothers and increased significantly with ∑PBDEs, ∑PCBs and ∑DDTs and not significantly with HCB in pups. This phenomenon is due to transfer of contaminants of their offspring, leading to their slight detoxification. Indeed, females lose relatively a little burden of contaminants during lactation. A likely explanation for this is that harp seal females lose relatively little weight during lactation comparatively to other phocid species. In spite of the production of high amounts of lipid-rich milk during lactation, females lose only 28% of their body weight (Kovacs et al. 1991), mainly blubber and associated contaminants. The average lost in weight by the lactating females is almost 37 kg (Kovacs et al. 1991), similar to lost weight observed in our study.

Data from the present study show that there is significant accumulation of PCBs, PBDEs and OCPs in harp seal pups as a result of lactational transfer of these compounds from the mothers. The time frame in which contaminants are transferred from mother to pup occurs. Exposure of pups to POPs during at early life stages may have toxic impacts on their developing endocrine, immune and neural systems. The impact of these contaminants on health and development of harp seal pups is currently unknown, but it is known that these persistent contaminants may produce subtle biological effects, even at low concentrations (Beckmen et al. 2003; Hall et al. 2003; Sørmo et al. 2005; Frouin et al. 2009a).

Table 2. Mean (± S.E.) concentrations (ng g^{-1} lipids) of PCB-, PBDE-, and pesticide groups in blubber, blood and milk of harp seals

Analyte	Pup blubber	Pup blood	Maternal milk	Maternal blubber	Maternal blood
4-Cl PCB	19.0 ± 9.1	19.2 ± 5.1	24.7 ± 7.8	24.4 ± 6.5	10.8 ± 7.2
5-Cl PCB	42.7 ± 20.1	62.7 ± 19.8	53.2 ± 13.8	96.2 ± 21.6	34.3 ± 17.3
6-Cl PCB	75.7 ± 28.2	176.4 ± 54.9	96.8 ± 27.8	368.4 ± 79.6	112.0 ± 36.1
7-Cl PCB	22.6 ± 8.7	79.6 ± 33.4	30.3 ± 10.9	226.1 ± 67.4	57.4 ± 44.9
∑PCB	160.2 ± 63.5	345.8 ± 118.5	214.1 ± 58.5	754.4 ± 171.9	220.9 ± 98.8
4-BDE	13.6 ± 6.1	20.4 ± 9.5	11.2 ± 5.0	22.4 ± 10.2	19.1 ± 13.9
5-BDE	3.7 ± 2.2	7.3 ± 3.8	3.0 ± 1.2	9.7 ± 4.2	5.7 ± 2.5
6-BDE	0.8 ± 0.5	3.1 ± 2.6	0.7 ± 0.3	4.4 ± 2.5	36.0 (q.l.) (0)
∑PBDE	18.8 ± 8.1	30.8 ± 11.5	15.0 ± 16.4	37.7 ± 16.1	24.8 ± 15.2
HCB	45.3 ± 28.2	47.4 ± 14.8	43.9 ± 16.5	35.1 ± 11.3	32.1 ± 19.0
∑DDT	139.8 ± 42.7	172.8 ± 37.9	148.1 ± 33.4	312.2 ± 72.0	102.0 ± 26.7
∑CHL	134.8 ± 31.1	151.3 ± 55.3	140.4 ± 34.6	300.2 ± 82.3	66.2 ± 12.9
Mirex	3.1 ± 2.4	21.3 ± 4.8	3.9 ± 1.4	13.4 ± 3.4	33.7 ± 13.3
HCH	31.0 ± 18.4	15.3 ± 11.6	34.1 ± 18.0	29.3 ± 12.7	5.3 ± 7.1

q.l. = quantification limit
∑PCB is the sum of PCB 8, 15, 18, 28, 31, 33, 44, 49, 52, 66, 70, 74, 87, 99, 101, 105, 110, 118, 123, 128, 138, 149, 151, 153, 156, 170, 171, 177, 180, 183, 187, 191, 194, 195, 199, 205,206, 208, 209.
∑PBDE is the sum of BDE 17, 29, 47, 49, 66, 99, 100, 153, 154.
∑DDT is the sum of 4,4'-DDE, 4,4'-DDD, 2,4'-DDT, 4,4'-DDT.
∑CHL is the sum of oxychlordane, γ-chlordane, *trans*-nonachlor, *cis*-nonachlor
∑HCH is the sum of α-HCH, β-HCH, γ-HCH.

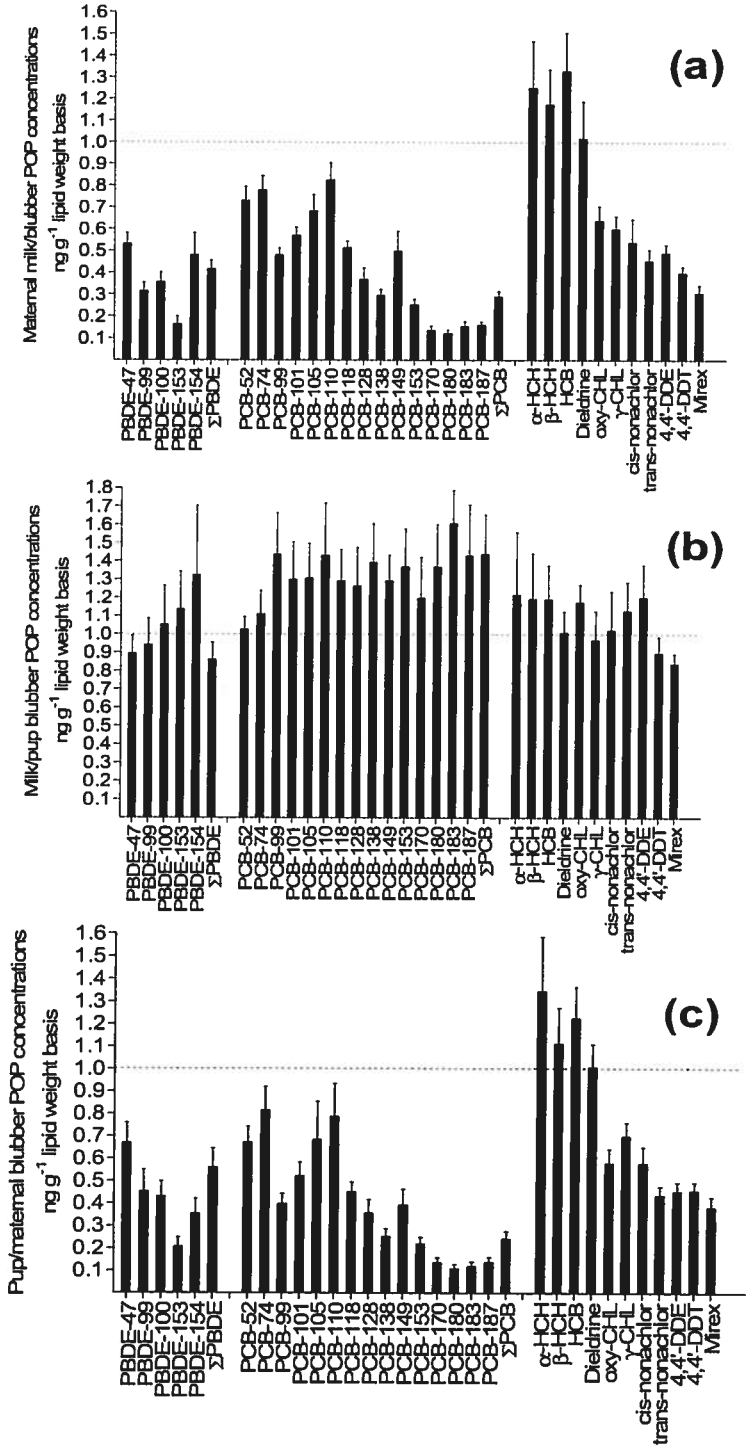

Figure 3. Milk concentrations of POPs relative to corresponding concentrations (ng g^{-1} lipid) in maternal blubber (a) and pup blubber (b) and pup blubber POP concentrations relative to corresponding (ng g^{-1} lipid) in maternal blubber (c) in harp seal mother-pup pairs. Data from early/mid and late lactation were combined.

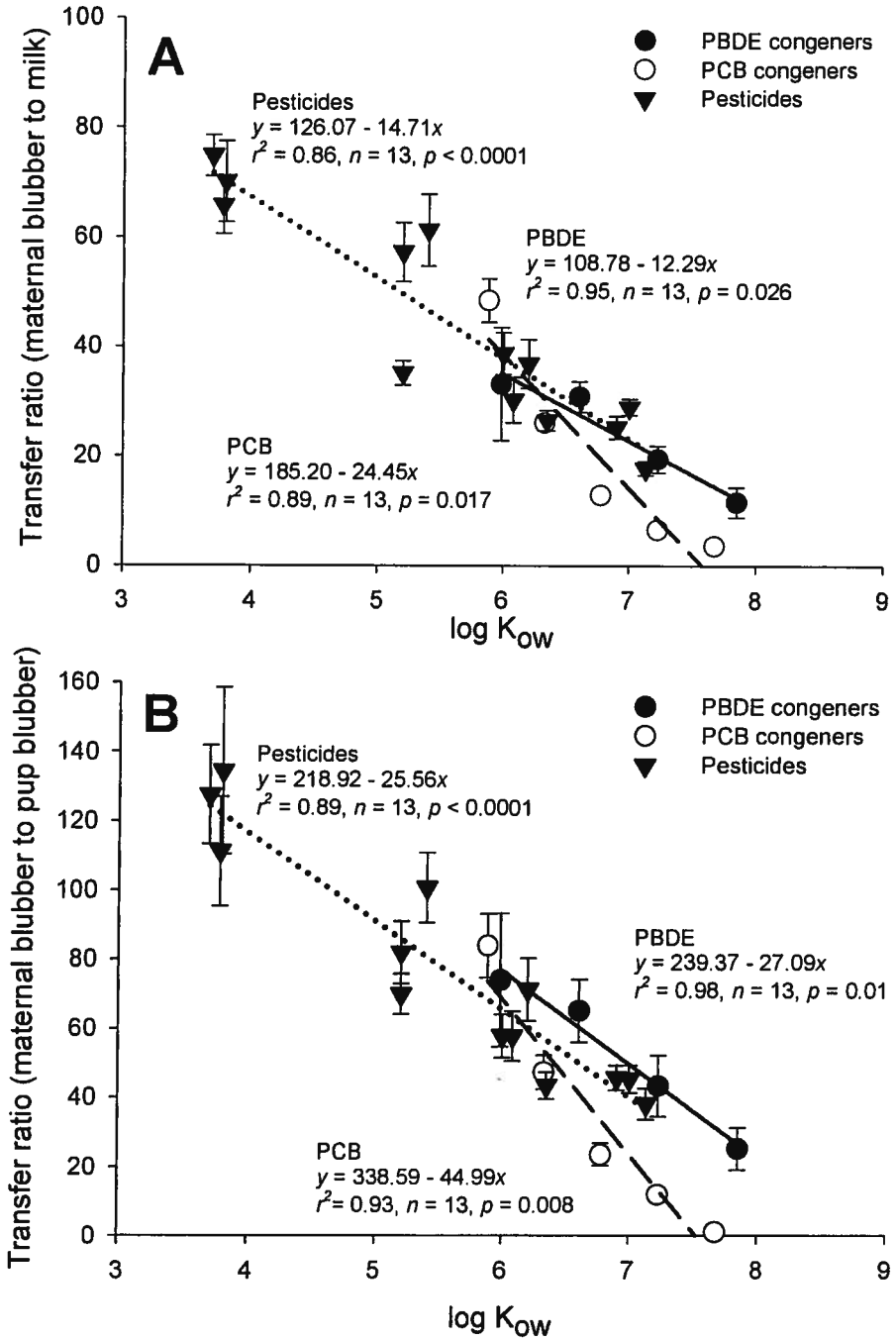

Figure 4. Relationship between the octanol/water partition coefficient (log K_{ow}) values and the transfer rate (%) of organochlorine pesticides, PCB congeners and PBDE congeners from maternal blubber into milk (A) or to pup blubber (B) in harp seals (*Phoca vitulina*) from Magdalen Islands. (Values of log $K_{o/w}$ were calculated from data of Finizio et al. 1997; Braekevelt et al. 2003). Data from early/mid and late lactation were combined.

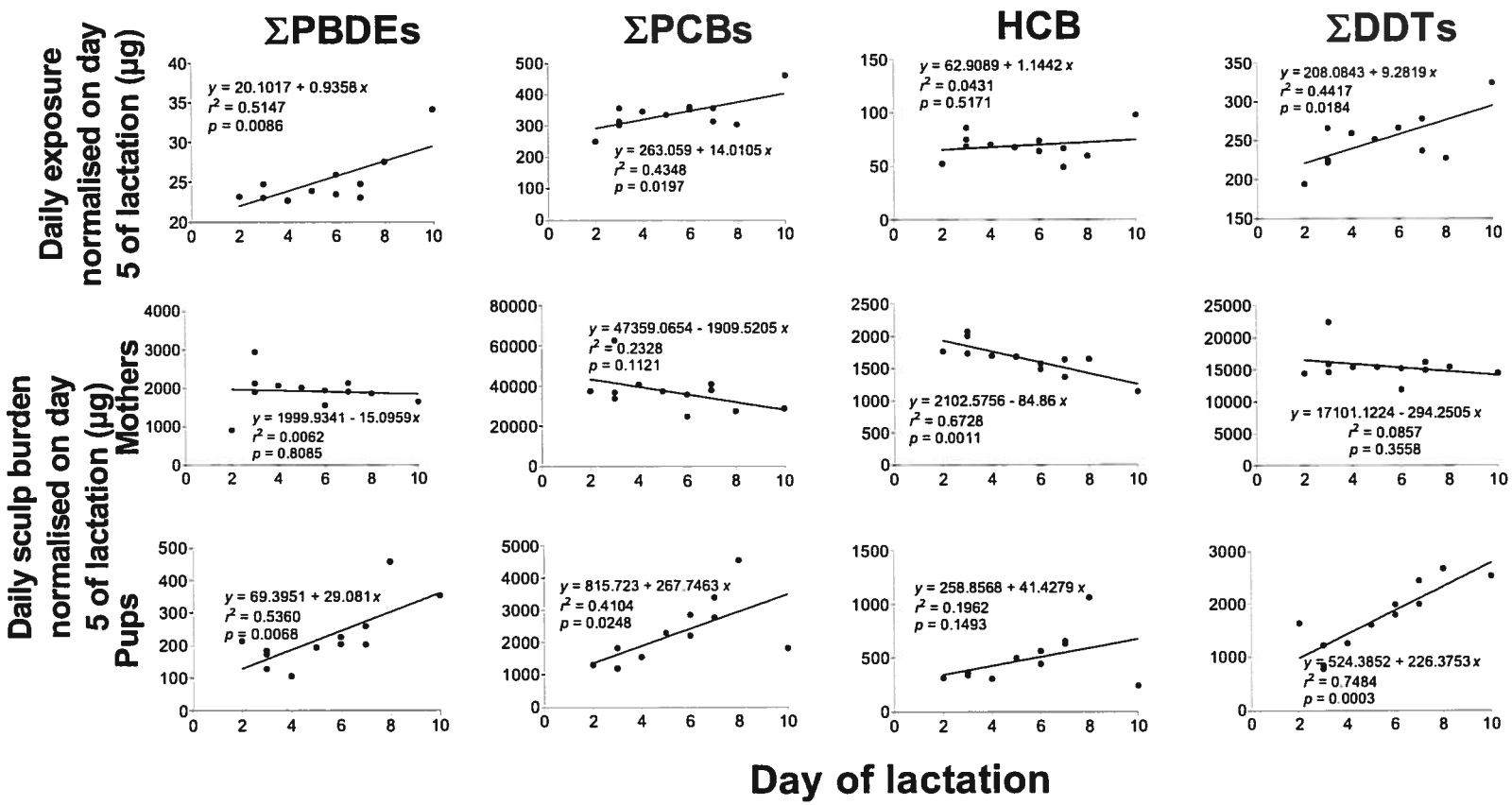

Figure 5. Contaminant exposure (ΣPBDEs, ΣPCBs ΣDDTs and HCB) experienced by individual harp seal pups and contaminant blubber burden in mothers and pups. Values were normalised on the basis of 5th day of lactation.

ACKNOWLEDGEMENTS

Fisheries and Oceans Canada (Maurice Lamontagne Institute, Mont Joli, QC; M.O.H. and M.L.) supported this work. This study was also supported by the Canada Research Chair in Environmental Immunotoxicology (M.F.). The authors thank the following individuals for their generous contributions with sample acquisition/analyses: Samuel Turgeon, Harrison MacRae, Steve Trottier, Tiphaine Brett.

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4.2. PBDE dans le sérum et le gras de nouveau-nés phoques commun, gris et du Groenland de l'Est du Canada

Article 3: *Héloïse Frouin, Michel Lebeuf, Mike Hammill, Becky Sjare, Michel Fournier. PBDEs in serum and blubber of harbour, grey and harp seal pups from Eastern Canada.* Mon rôle a consisté à faire l'échantillonnage, les analyses, interpréter les données et écrire cet article à titre d'auteur principal.

RÉSUMÉ

Le sérum et le gras de nouveau-nés chez trois espèces de phoques (commun, gris et du Groenland) de l'Estuaire et du Golfe du St-Laurent ont été analysés pour les polybromés diphényles éthers (PBDE). Les concentrations de PBDE (normalisées sur une base lipidique) dans les tissus de nouveau-nés de phoque commun vivant dans l'Estuaire du St-Laurent sont au moins 5 fois plus élevées que ceux de la colonie localisée dans le Golfe, indicant que la contamination aux PBDE dans l'estuaire, provenant des apports fluviaux, est significativement plus élevée que dans le Golfe. Sans tenir compte de l'espèce ou de la localisation, les concentrations (normalisées sur une base lipidique) de ∑PBDE dans le sérum ou le gras sont similaires, suggérant que ces deux matrices peuvent être utilisées pour le suivi des PBDE chez les nouveau-nés. Cependant, les congénères les plus bromés, qui sont des composants mineurs de la ∑PBDE, sont retrouvés en plus grande quantité dans le sérum comparativement au gras, ce qui suggére pour ces composés des affinités de liaison avec certains constituants du sérum. Les nouveau-nés phoques communs, gris et du Groenland qui sont nées dans le Golfe du St-Laurent ont des niveaux similaires de PBDE. Cette étude montre que les phoques nouveau-nés ont une exposition substantielle aux contaminants bromés à un stade critique de leur développement et que l'Estuaire du St-Laurent est un contributeur important de leur contamination aux PBDE.

ABSTRACT

Serum and blubber of pup harbour (*Phoca vitulina*), grey (*Halichoerus grypus*) and harp (*Phoca groenlandica*) seals from the Estuary and the Gulf of St. Lawrence were analyzed for polybrominated diphenyl ethers (PBDEs). PBDE concentrations (on lipid weight basis) in tissues of harbour seal pups inhabiting the St. Lawrence Estuary were about 5 times higher than in those from a colony located in the northern Gulf, indicating that PBDE contamination in the Estuary, likely from fluvial inputs, is significantly higher than in the Gulf. Regardless of seal species or locations, lipid normalized concentrations of ΣPBDEs in serum or blubber were similar suggesting that either matrices could be used to monitor PBDEs in pups. However, highly brominated congeners, which are minor components of ΣPBDEs, are encountered in higher amounts in serum than into blubber, which suggest enhanced bonding affinities of these congeners to certain constituents in serum. Harbour, grey and harp seal pups born in the Gulf of St. Lawrence had similar levels of PBDEs. This study shows that seal pups have a substantial exposure to brominated contaminants at a critical developmental stage and that the Estuary of St. Lawrence is an important contributor to their PBDE contamination.

Key words : PBDEs, Marine mammals, pinnipeds, St. Lawrence Estuary, St. Lawrence Gulf

INTRODUCTION

Brominated flame retardants (BFRs) are man-made chemicals used in a large variety of flammable polymers. With the increasing usage of polymeric materials in construction, automotive, electronic and computer equipment, global market demand for the use of BFRs has grown substantially during the last decade [1]. Among BFRs, PBDEs (Polybrominated diphenyl ethers) have been used since the 1980s in North America [2] and continue to be used though under increasingly restricted conditions [3]. PBDEs are blended but not chemically linked to polymers [1] and thus may migrate more easily from products to environment during production, use and recycling of products containing PBDEs as well as disposal in domestic waste, landfills and incineration [4, 5].

Recent studies have shown that PBDEs are ubiquitous in the freshwater and marine environments worldwide and bioaccumulate in food webs [6, 7]. In the St. Lawrence marine ecosystem (SLME), which includes the St. Lawrence Estuary and the

Gulf of St. Lawrence (Canada), only a few studies have reported PBDE concentrations in marine organisms, mainly marine mammals [8-11].

PBDE are similar in behaviour (hydrophobic, lipophilic, thermally stable) and toxicity to the well-studied contaminants polychlorinated biphenyls (PCBs). Marine organisms of the St-Lawrence Estuary (SLE) food web are believed to be exposed to organochlorine contaminants originating from fluvial inputs as the SLE is located downstream of the Great Lakes-St. Lawrence River and receives the waters from one of the most industrialised regions of the world. Lebeuf and Nunes [12] demonstrated previously that at least 70% of the total burdens of persistent organochlorine pollutants, such as PCBs, dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB) and mirex, in the SLE sediments were issued from upstream fluvial waters. However, no data have been published describing sources of PBDEs in or to the SLME.

The SLME is inhabited by four species of seals. These include several colonies of harbour seals (*Phoca vitulina*), including one that is a permanent resident of the SLE [13]. Grey seals (*Halichoerus grypus*) summer in the SLE in summer but overwinter in the Gulf of St. Lawrence or on the Atlantic coast of Canada [14]. Harp (*Phoca groenlandica*) and hooded (*Cystophora cristata*) seals winter in the SLME, but summer in Arctic waters [15]. PBDEs concentrations have only been reported in adult harbour seals from the SLE [8] and pup and adult hooded seals [11], however there is a lack of data on PBDE contamination for harp and grey seals. Recent studies have shown that organochlorine pollutants such as PCB and mirex originating from fluvial inputs [16] are found at high concentrations in harbour seals that are resident in the estuary whereas POPs such as chlorobornanes (CHBs) originating from the atmosphere [17] are relatively low in these harbour seals from the estuary but high in hooded seals compared to other seal species from the SLME. Thus, it is believed that PBDE data in tissues of seal species from SLME should help to identify the main source of this contaminant.

Blubber is the most common tissue analysed to assess POPs. However, the collection of blood is a less invasive sampling method and is currently being evaluated as an alternative approach. In adult seals, blood has proven to be a poor substrate to characterize POP contamination [18], but a similar evaluation focusing on seal pups has not been carried out.

Suckling neonate seals, during the short lactation period (4-17 days) [19, 20], are at a critical stage of neurological and immune system development and are particularly sensitive to adverse effects of lipophilic contaminants even at low levels of exposure

[21]. PBDE are known to be present in milk, rich in lipids, and transferred to seal offspring [11]. Although limited, some laboratory studies have indicated that certain PBDE congeners may be hepatotoxic, immunotoxic and have endocrine disrupting effects (review by [22] and [23]). In addition, Hall et al. [24] suggested that PBDEs may function as endocrine disrupters in grey seal pups.

The objectives of this research were as follows: 1) determine the levels and distribution profiles of PBDEs in serum and blubber tissues of three different species of seal pups in the SLME; 2) assess if serum PBDE contamination in pups is representative of the contamination in blubber for each species; 3) consider whether differences in PBDE contamination among these seal pups are best explained by location of core habitat relative to contaminant sources, residency time in the SLME, migration routes or diet of the mothers.

MATERIAL AND METHODS

Sample collection and storage

Six harbour seal pups (Pv Bic) were live captured in the SLE (Bic Island near Bic National Park) in May of 2007 and six other pups (Pv NF) in the northern Gulf of St. Lawrence (St. Paul's inlet, west coast of Newfoundland) in May of 2008 (Fig. 1). Six grey seal pups (Hg) were live captured on ice pack in early January of 2008 in the southern Gulf of St. Lawrence, near Amlet Island in the Northumberland Strait and on Hay Island off the east coast of Cape Breton Island. In March 2008, six harp seal pups (Pg) were live captured on the pack ice near the Magdalen Islands in the northern Gulf of St Lawrence. Prior to sampling, animals were weighed (to ±0.5 kg), tagged, and their sex noted. Standard length and axillary girth were measured; the seal was positioned on its ventrum with its head and vertebral column in a straight line. Body condition was assessed by calculating a condition index for each seal (axillary girth/standard length x 100; [25]). Pups were captured throughout the lactation period and aged based on body sharp, condition of the pelage and mass. Additional information on these samples, including details on the formulas used to calculate age, is reported in the supporting information (Table S1).

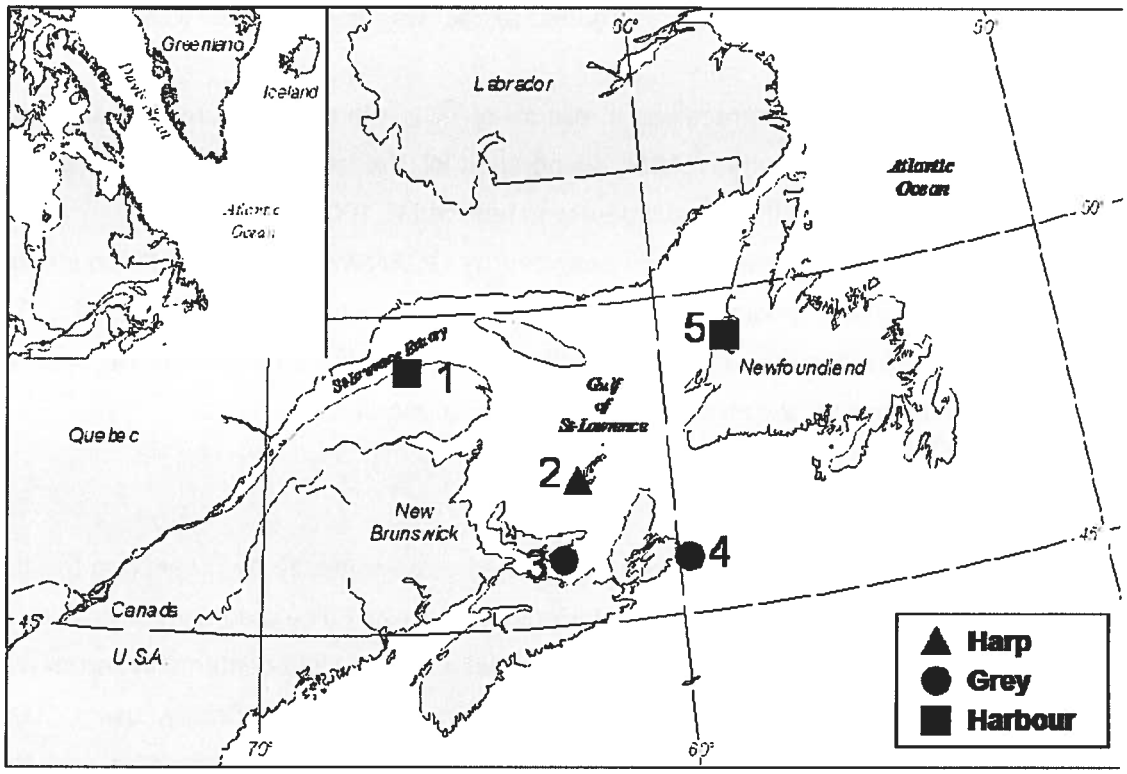

Figure 1. Location of capture site for seal pups collected in the St. Lawrence Estuary and the Gulf of St. Lawrence. 1 : Bic Island (near Bic National Park); 2 : Madgalen Islands; 3 : Amet Island; 4 : Hay Island; 5 : Newfoundland.

A sample of the entire subcutaneous blubber layer was taken from each pup using a 6 mm biopsy punch. Samples were wrapped in solvent rinsed aluminium foil, placed in a sealed plastic jar, were kept in a cooler with ice packs for up to 10 h in the field and then stored at -20℃ until analysis. Blood samples from each pup were taken from the extradural intravertebral vein using Vacutainer tubes (Becton-Dickinson, New Jersey, USA), and then kept in the field at 4℃ until processing (within 10 h). Blood samples were centrifuged and the sera were frozen at -20℃ until analysis. All research was approved by the Animal Care Committees of Fisheries and Oceans Canada.

Chemical analysis of PBDEs

The methodology for extraction, cleanup and quantification of target analytes (PBDEs) in blubber samples by GC/MS/MS, as well as QA/QC procedures are reported elsewhere [10], but briefly presented below. Between 0.06 and 0.27 g of blubber was dried with ~ 20 g anhydrous Na_2SO_4, spiked with surrogates $^{13}C_{12}$ PBDEs (BDE-47, -99

and -153). A fraction (10%) of the extract was used to lipid determination. The rest of the extract was cleaned and eluted then cleaned extract was concentrated to a final volume of 100 µL.

Seal serum samples received a mixture of $^{13}C_{12}$ internal standards extracted with hexane:methyl tert-butyl ether. After evaporation of the elution solvent the recovery standard was added and the final volume was reduced to 100 µl.

Seal blubber and serum were analyzed by GC/MS/MS for 39 PBDE congeners, from di to hepta-BDE homolog groups. The limit of quantification was established at 5.71 ng g^{-1} lipid for blubber and 13.15 ng g^{-1} lipid for serum. More details on the chemical analysis in blubber and serum can be found in the supporting information.

Lipid determination

Lipid contents in blubber were determined gravimetrically by measuring the total non-volatile solvent extractable material. Percent lipid was calculated using the following equation: % lipid = (mass of lipid/mass of sample) x 100%. Lipid contents in serum were measured using the colorimetric technique of Frings et al. [26]. Briefly, using 100 µl serum from each seal sample, lipids were hydrolyzed with sulphuric acid and then reacted with phosphor-vanillin, producing a chromophore (absorption maximum 540 nm). Sample absorption was determined using ultraviolet/visible (UV/Vis) spectroscopy (Thermo Electron Instruments MULTISKAN ASCENT), and lipid concentration was quantified using a linear, five-point calibration curve of olive oil standards that bracketed all analyzed samples.

QA/QC

One procedural blank and one Standard Reference Material (SRM 1947) were included with every 10-blubber sample analysis for PBDE concentrations and lipid determinations. A blubber sample (SRM 1945) from a pilot whale (Globicephala sp.) was obtained from the National Institute of Standards and Technology (NIST) and used as the standard reference material. Repeated analysis of SRM 1945 (n = 5) indicated that the precision of the method, reported as the coefficients of variation (CV), for all detected PBDEs varied between 9 and 20 % of the average values. The analytical accuracy of the results was also determined using the certified reference material SRM 1945 (n = 5). The levels of most detected PBDE congeners (28, 47, 49, 66, 99, 100, 153, 154, 155 and

183) were within 20% of expected values [27]. Nine major congeners of PBDEs (BDE-17, -28, -47, -99, -100, -138, -153, -154 and BDE-155) were quantified in this study.

The serum samples were analysed in batches of 12, which included one blank, one blank intentionally fortified, one human serum sample (SRM 1589a [28]) used as the standard reference material and nine samples. The CV of replicate analysis of SRM 1589a (n = 5) and blank fortified (n = 5) were respectively within 22% and 9 % of the average values, indicating that the precision of the method was satisfactory. The analytical accuracy of the results was also determined using the certified reference material SRM 1589a (n = 5). The levels of most PBDE congeners measured (47, 99 and 100) were within 24% of expected values. Four major congeners of PBDEs (BDE-47, -99, -100 and -153) were quantified in this study. For blubber and serum samples, all data were corrected for the recovery of the surrogates that was in average 99 ± 13 %.

Data analysis

Data compilation and statistical analyses were performed using Microsoft excel 2003 and STATISTICA for windows (version 7.0, StatSoft Inc, 1995), with a significance level of $p < 0.05$. Concentrations of total PBDEs and all congeners reported individually are in nanograms of analyte per gram of sample and lipid normalized for comparison. As no significant relationships were observed between age and concentrations within any sample group, data were not age-normalized. The distribution of each variable was examined with the Kolmogorov-Smirnov and Shapiro Wilk normality tests. Differences of PBDE concentration between sampling groups were investigated using a single factor analysis of variance (ANOVA) followed by a Tukey's post-hoc comparisons test. PBDE concentrations, age, blubber lipid content, serum lipid content and condition data used in ANOVA tests were not logarithmic transformed. Serum/blubber ratio of lipid normalized PBDE concentrations were calculated for each congener and each species or locations. When concentrations were below the detection limit (e.g., BDE-154 in serum) values of the method quantification limit were used. Since serum/blubber concentration ratios were normally distributed, two-tailed t tests were used to determine if ratios were different than unity (value 1).). All statistical tests used a significant level of $p < 0.05$.

RESULTS AND DISCUSSION

PBDE levels and distribution profiles in serum and blubber of seal pups

PBDEs were found in all blubber and serum samples analysed irrespective of the species or the locations. Only six congeners were present as major compounds (BDE-47, -99, -100, -153 and -154). A Tukey's post-hoc comparison revealed that seals could be separated into two groups based on their respective ΣPBDE (all PBDEs detected) mean concentrations in blubber (± standard error). ΣPBDE in harbour seal pups from Bic (539.6 ± 61.0 ng/g lipid weight (l w) being more contaminated than harbour seals from St. Paul (96.8 ± 16.3 ng/g l w), grey seals (70.8 ± 8.0 ng/g l w) and harp seals (22.0 ± 3.4 ng/g l w) (Fig. 2). Two groups were also observed based on their respective ΣPBDE mean concentrations in serum: harbour seals from Bic (585.2 ± 70.0 ng/g l w) were more contaminated than harbour seals from St. Paul (83.7 ± 21.6 ng/g l w), grey seals (41.1 ± 7.2 ng/g l w) and harp seals (30.5 ± 5.4 ng/g l w).

BBDE-47, -99, -100 and -153 were detected in all blubber samples, but BDE-154 was below the detection limit in serum samples from grey seals (Fig. 3). PBDE distribution profiles in seals were compared to the penta-DBE mixture (DE-71) [29]. Major congeners of PBDE were BDE-47 in seals and BDE-47 and -99 in pentaBDE. Metabolism of BDE congeners could explain these deviations, considering that in vivo debromination of BDE-99 to BDE-47 has been demonstrated in fish [30]. It cannot be excluded that BDE-47 and other low brominated PBDEs could result from the debromination of highly brominated congeners found in octa and deca-BDE-mixtures. BDE-47 in blubber accounted for more than 83% of sum of PBDEs in harbour seals from Bic, 81% in harbour seals from St. Paul, 78% in grey seals and 74% harp seals. Penta-BDE and hexa-BDE contributed from 14 to 23% and from 3 to 7% of ΣPBDEs, respectively (Fig. 3). In serum, BDE-47 accounted for more than 83% of ΣPBDEs in harp seals, 76% in harbour seals from St. Paul and 74% in both harbour seals from Bic and grey seals. Penta-BDE and hexa-BDE contributed from 6 to 11% and from 2 to 6% of ΣPBDEs, respectively. BDE-47 and -99 are generally dominant in marine mammals worldwide [31] and suggest an exposure to the technical penta-BDE [29]. These results are similar to those of a previous study [32], which demonstrated that in stranded, recently deceased harbour seal pups, BDE-47, penta-BDE and hexa-BDE accounted for 73%, 18% and 5%, of ΣPBDEs content in blubber, respectively. This is in agreement, with a previous study on grey seal mother-pup pairs [9], where tetra-BDE (BDE-47 comprised over 90% of the tetra-BDE), penta-BDE and hexa-BDE represented respectively about 79%, 14% and 5% of ΣPBDEs in blubber of pups.

Limited data of PBDEs have been reported in seal pups from the Northern hemisphere (Table 1). PBDE mean concentrations measured in blubber of harbour seals from Bic Island and St. Paul were lower than those reported in harbour seals from eastern Maine-Long Island area (New York) [32] and from the southern California coast [33] and similar to these detected in harbour seals from the San Francisco bay area [34]. On the other hand, PBDE levels measured in serum of harbour seals from the Estuary and the Gulf of St. Lawrence were higher than those reported in whole blood of harbour seal pups from California [35]. Levels of PBDEs reported in this study in grey seals were higher than for animals collected in Sable Island (East coast of Canada) [9] but were about 4 times lower than those sampled in Europe [24, 36]. To our knowledge, there are no other data available on PBDE concentrations in grey seal pup serum or in harp seal pup tissues. In the case of hooded seal pups in the Gulf of St. Lawrence, Wolkers et al. [11] reported that levels of PBDE concentrations in blubber and plasma were 1.5 and 5 times higher than for harp seals. Thomas et al. [37] demonstrated than there was no statistical difference, for PBDE concentrations between serum and plasma on an extracted lipid weight basis. PBDE concentrations in pups of all species from the SLME were lower comparatively to other regions in Northern Hemisphere. It is important to note that previous data suggested that PBDE levels were increasing in marine mammals between the 1980s and the 2000s [34, 38]. Thus, comparison between the results reported in this work and in previous studies is likely biased by temporal trends and must be interpreted with caution.

Accumulation of PBDEs, as demonstrated in this study, in marine mammal fetuses and pups through gestation and lactation suggest potential toxicological implications for them. Health risks from exposures to PBDEs in mammals include impacts on thyroid hormone levels, neurobiological development and fetal toxicity [22].

Is serum a reliable tissue to represent PBDE contamination in pups?

Serum/blubber ratios, which examine the relationship between PBDE concentrations in serum and blubber, were reported for each PBDE congener and ΣPBDEs for each species and locations (Fig. 4). The mean ratios of ΣPBDEs were not statistically different from unity (value 1) for seal pups analyzed by species or by location. The mean ratio for BDE-47 was not significantly different than unity except in harp seals. The highly substituted PBDEs (BDE-99, -100, -153 and -154) had ratios statistically higher than unity, indicating enrichment of these congeners in serum versus blubber.

Similar observations were reported by Wolkers et al. [11], the mean plasma/blubber ratios for tetra-BDE (dominated by BDE-47), penta-BDE (dominated by BDE-99 and -100), hexa-BDE (dominated by BDE-153 and -154) and ΣPBDEs were 2.8, 14.3, 12.2 and 4.7, respectively. Finally, BDE-154 was detected in a few serum samples and the use of limit of quantification for non detected concentrations may underestimate BDE-154 serum/blubber concentration ratios.

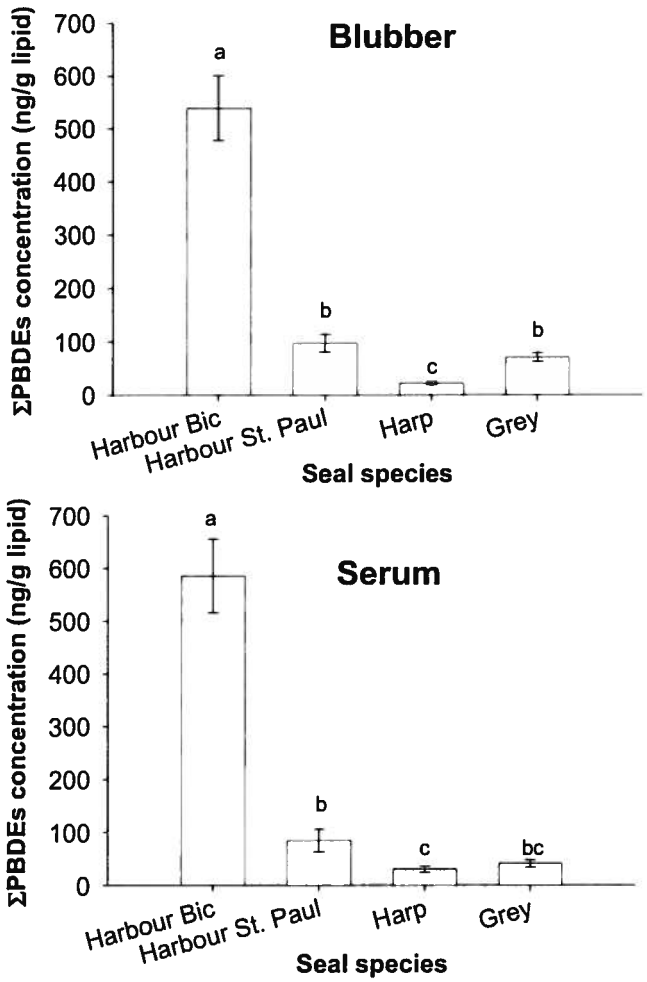

Figure 2. Mean concentration (pg/g lipid weight) of ΣPBDEs (± standard error) in blubber (a) and serum (b) seal pups from eastern Canada. Different letters indicate significant differences between seal species ($p < 0.01$).

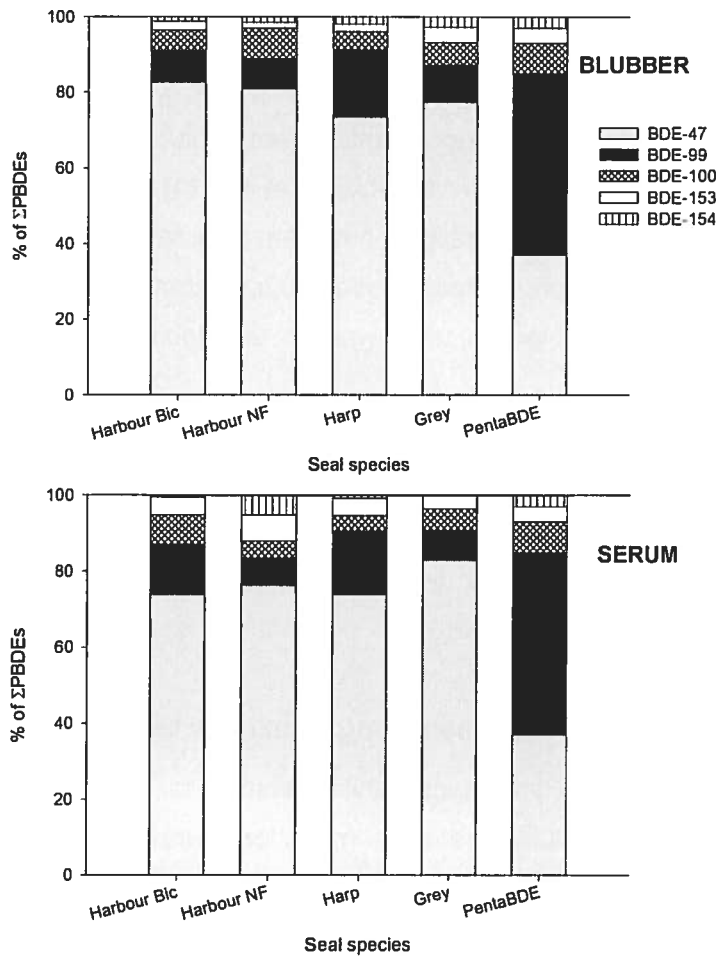

Figure 3. Mean contribution of individual PBDEs relative to ΣPBDE concentrations in blubber (a) and serum (b) pup seal species from eastern Canada compared to the technical PentaBDE product.

The PBDE profile changes from one tissue to the other as a result of the physicochemical properties of the different congeners and of the lipid composition of the tissue considered in seal pups. The lipid composition in blood of young seals consists mainly of non-polar lipids (triglycerides and cholesterol esters) [39] in which highly brominated congeners, which are more lipophilic and less polar, may be more soluble than in non-polar triacylglycerol blubber lipid during lactation period. Moreover, prodigious reserves of triacylglycerol accumulate in blubber of seal pups during the first few days of neonatal development; the high levels attained at this time are maintained during subsequent development [40]. Thus, PBDEs transferred by lactation could be retained by blood and deposited in lower quantities in the blubber.

In the context of a monitoring program, selection of the matrice is critical but appears to be different between pup and adult seals. Among pups both blood and blubber represent accumulation of pollutants over a short time period while in adults, blood levels represent short term accumulation of pollutants whereas blubber integrates the accumulation of pollutants over longer durations. Wolkers et al. [11] demonstrated that no correlation existed in ΣPBDEs concentrations between plasma and blubber, suggesting that plasma of adult and pup hooded seals overestimated ΣPBDEs. Moreover, Lydersen [18] concluded that blood plasma was an unreliable indicator for contaminant exposure in adult harp seals.

The present study demonstrates that serum of lactating pups can be used to represent their blubber concentration of ΣPBDEs or BDE-47 which is the most dominant PBDE congeners measured in seals. However, serum clearly overestimates concentrations of the highly brominated, but less dominant, congeners accumulated in blubber.

Explaining the PBDE contamination of seal pups among species or locations

Several factors such as the location of important habitat relative to contamination sources upstream, residency time in the SLME, seasonal migration routes or the diet of seals can affect PBDE levels in their tissues.

Location

Harbour seals feeding opportunistically [41] and the prey selection is in relation to variations in prey abundance [42]. Bowen and Harrison [43] shown that the harbour seal diets in two inshore habitats of Atlantic Canada (lower Bay of Fundy and along the northeastern coast of Nova Scotia) were similar and dominated by Atlantic herring (*Clupea harengus*). In the case of harbour porpoises (*Phocoena phocoena*) from the Estuary or the Gulf of St. Lawrence, there is no evidence for strong regional differences in diets (Fontaine et al., 1994), which were dominated by Atlantic herring. Unfortunately, little information exists regarding the diet of harbour seals both colonies remain in the SLME the entire year (Bic and St. Paul). So, we suggest that diets of harbour seals from the Estuary (Bic) or the Gulf (St. Paul) of St. Lawrence are also similar and based on what we know about PBDE levels in prey items, we can confirm that diet is not a factor in the explaining the differences in PBDE levels observed and the location of the two colonies relative to contamination sources appears to be the most important factor.

Table 1. Mean concentration ± standard deviation (ng/g lipid) of five major PBDEs, BDE-47, 99, 100, 153, 154 in blubber and blood of seal species from Northern Hemisphere

Seal species	location	Sampling period	tissues	sex	*n*	age (yr)	lipids (%)	47	99	100	153	154	ΣPBDEs	ref
harbour seal *Phoca vitulina*	St. Lawrence Estuary	2007	blubber	1F, 5M	6	1-10 days	65 ± 23	399.9 ± 38.4	40.6 ± 5.9	25.9 ± 2.0	11.1 ± 3.4	6.1 ± 4.0	539.6 ± 61.0	This study
	St. Lawrence Gulf	2008	blubber	3F, 3M	6	3-9 days	58 ± 9	70.8 ± 11.3	7.1 ± 1.6	7.0 ± 1.8	1.4 ± 0.3	1.3 ± 0.4	96.8 ± 16.3	This study
	San Francisco	1992	blubber	n.d.	1	fetus	66.9	277	85.6	12.4	10.5	44.6	430	[34]
	From eastern maine to Long Island, New York	1991-2005	blubber		13	pups	59.1	2970	308	308	99	41.7	3645	[32]
	Southern California	1994-2006	blubber	3F, 5M	8	pups	57 ± 25						2430 ± 2250	[33]
	St. Lawrence Estuary	2007	serum	1F, 5M	6	1-10 days	1.2 ± 0.4	429.2 ± 48.1	76.4 ± 13.2	45.3 ± 3.1	26.6 ± 4.4	3.7 ± 3.7	585.2 ± 69.8	This study
	St. Lawrence Gulf	2008	serum	3F, 3M	6	3-9 days	0.8 ± 0.2	63.9 ± 13.2	5.9 ± 3.2	3.8 ± 2.5	5.7 ± 2.1	4.4 ± 2.1	83.7 ± 21.6	This study
	California	2001-2002	blood	F & M	17	pups							0.07-3.02*	[35]
grey seal *Halichoerus grypusu*	St. Lawrence Gulf	1995	blubber		5	14-24 d.		39.5 ± 21.2	3.9 ± 2.5	2.7 ± 1.4	1.2 ± 0.7	1.3 ± 0.9	50 ± 26.8	[9]
	St. Lawrence Gulf	2008	blubber	3F, 3M	6	0-4 days	64 ± 17	40.9 ± 5.2	5.2 ± 1.3	3.1 ± 0.5	2.2 ± 0.6	1.4 ± 0.4	70.8 ± 8.0	This study
	St. Lawrence Gulf	2008	serum	3F, 3M	6	0-4 days	1 ± 0.2	34.1 ± 6.3	3.2 ± 1.2	2.4 ± 0.9	1.4 ± 1.0	<0.1	41.1 ± 7.2	This study
	Farne Islands, UK	1998-1999	blubber	F & M	54	pups							222.6± 150.4	[24]
	North Sea	1998-2000	blubber	F & M	110	pups		210	16	21	11	8	290	[36]
harp seal *Phoca groenlandica*	St. Lawrence Gulf	2008	blubber	3F, 3M	6	1-6 days	72 ± 8	14.6 ± 2.5	3.5 ± 0.9	0.9 ± 0.2	0.4 ± 0.2	0.4 ± 0.1	22.0 ± 3.4	This study
	St. Lawrence Gulf	2008	serum	3F, 3M	6	1-6 days	1.5 ± 0.3	22.5 ± 4.2	5.1 ± 0.8	1.2 ± 0.4	1.4 ± 0.9	0.3 ± 0.3	30.5 ± 5.4	This study
hooded seal *Cystophora cristata*	St. Lawrence Gulf	2001	blubber		6	pups		25.3	1.2	1.9	1.6	0.1	31.9	[11]
	St. Lawrence Gulf	2001	serum		6	pups							149.2	[11]

n.d.: not detected; * : wet weight (ng/g whole blood); # : BDE47 + BDE99

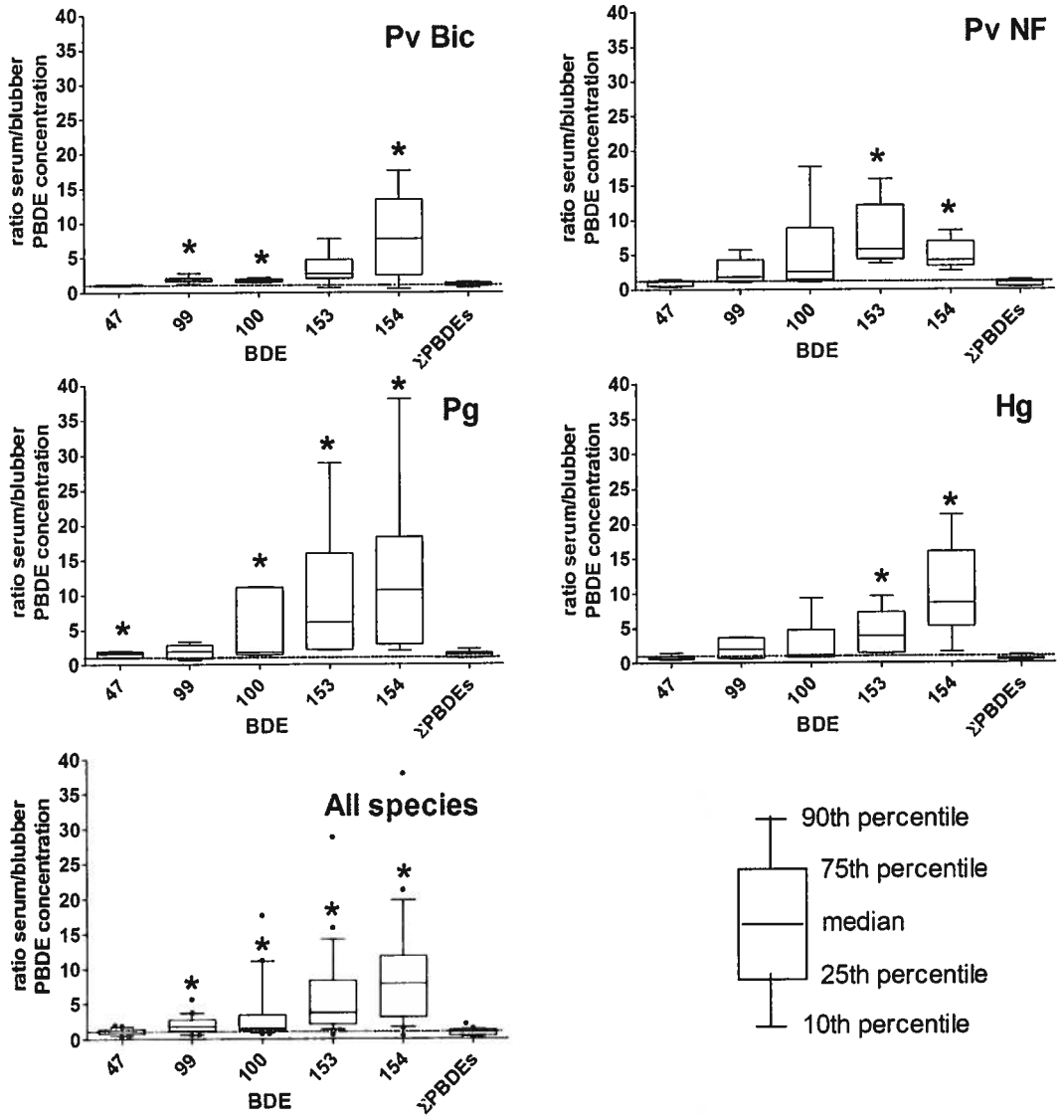

Figure 4. Serum concentrations of PBDE congeners relatively to corresponding concentrations (ng g^{-1} lipid) in blubber in harbour seal pups from Bic (Pv Bic), in harbour seal from Newfoundland (Pv NF), in harp seals from Magdalen Islands (Pg) and in grey seals from Amet and Hay Island (Hg). * ratio ≠ 1 (two-tailed t test, $p < 0.05$).

Concentrations of PBDEs in blubber and serum harbour seal pups from the Gulf of St Lawrence (St. Paul) were much lower than the levels observed in pups collected from the SLE (Bic). This is in agreement with the trend for PCBs in harbour seals

reported by Shawn et al. [44], which showed decreasing concentrations as a function of distance from sources. The spatial distribution of PBDEs is believed to be related to proximity to urban and industrial centers [45]. For instance, PBDEs enter the ecosystems through recycling and/or disposal of wastes containing PBDE [31], manufacturing output and fluvial deposition from tributaries [46] and wastewater treatment plants [47]. In this study, an urban rural-remote decreasing gradient was revealed for PBDEs in harbour seals.

In addition to urbanisation, atmospheric deposition could be a major source of PBDEs to aquatic systems [48, 49] including the Great Lakes [50]. Continental distribution patterns reported for atmospheric PBDEs in North America [51], indicate an increasing atmospheric gradient of PBDEs towards the north. Considering that both harbour seal colonies are located at approximately the same latitude (Fig. 1) but exhibit different levels of PBDE contamination, it seems that the amospheric contribution of PBDEs to the SLE is relatively limited compared to fluvial sources. This is supported by the results of contamination in seals from the SLME by toxaphene, a class of chemical known to enter the SLME by atmosphere transport [52]. Comparison of toxaphene concentrations in seals revealed that grey and harbour seals (from the Estuary) were less contaminated than harp and hooded seals [17]. On the contrary, large difference in contamination in seals from the Estuary compared to those from the Gulf were observed for chemical such as Mirex, known to enter in the SLE by fluvial inputs [16].

Residency time and habitat use (distant from source of contaminants)

Reduced residency time in SLME, moderate (grey seal) to highly (harp seals) seasonal migratory behaviour and geographic variation in diets make it much more difficult to explain PBDE levels in grey and harp seals. However, relevant data on residency time in combination with habitat use of these seal species while they are in the SLME is available and useful. After pupping in January-February, grey seals disperse from the southern Gulf of St. Lawrence to the north and to the east. They remain in offshore areas until moulting in May-June. Animals continue to disperse during July-September before returning to the southern Gulf in late fall for breeding [14]. Harp seals are highly migratory [53]. The Gulf herd breeds near the Magdalen Islands in the middle of the Gulf of St. Lawrence. [53] at the end of February. Adults then assemble north of their whelping patches to undergo the annual molt. The migration then continues north to Arctic summer feeding grounds. In late September, after a summer of feeding, nearly all

adults migrate southward along the Labrador coast, usually reaching the entrance to the Gulf of St. Lawrence by early winter. Consequently, both species are spending time foraging in Gulf. Concentrations of ∑PBDEs in harp seal pups from the Gulf of St. Lawrence were significantly different than those reported in harbour seal pups from Gulf of St. Lawrence (Fig. 2), whereas concentrations of ∑PBDEs in grey seal pups were similar to those in harbour seal pups. Extending the comparison by using data reported by Wolters et al. [11] reveals that PBDE concentrations in blubber of hooded seal pups from Gulf of St. Lawrence were lower than harbour and grey seal pups from Gulf but similar to those of harp seal pups reported in the present work. However, PBDE concentrations in plasma hooded seal (Table 1) [11] were higher than those in harp, grey and harbour seals from Gulf of St. Lawrence. No statistical differences existed in the contaminant levels or types of congeners between grey seals from Amlet Island vs Hay Island.

Migration routes/path:

The seasonal migration routes of grey and harp seals are relatively well documented; however, less is known about the contaminant profiles of seal prey along these migration routes and what contribution atmospheric born PBDE contaminant make presently there are no data to address these knowledge gaps. Even if we don't know the contamination level of prey and the seal diet composition all the way along the "migration path" we are fairly certain it is not contaminated to the level seen in the Estuary and Gulf.

Harbour seals are relatively sedentary throughout the year. Grey seals are found in winter on the ice in the southern Gulf of St.-Lawrence dispersing in summer along the northeastern United States, the Nova Scotia and Newfoundland coasts and throughout the Gulf of St. Lawrence [14, 54]. Harp and hooded seals have the same migration pattern and share their time between the Estuary, the Gulf of St. Lawrence and the Arctic. During their time outside the St. Lawrence, harp seals migrate north to Davis Strait and Baffin Bay and the western coast of Greenland, while hooded seals are seen along the eastern and the western coast of Greenland [15].

Diet

Differences of diet among seal species is known to influence their contamination by organic contaminants, because these compounds usually persist in the environment and are highly lipophilic, leading to their biomagnification in food webs [17]. For adults,

the main route of exposure is through diet, reflecting various inputs (local point and non-point sources and/or long-range fluvial transport), whereas young animals are exposed to PBDEs through placental and lactational transfer, which is selective because of differences in lipophilicity and molecular weight between compounds [9]. Like other POPs, a significant fraction of PBDEs found in juvenile marine mammals are transferred from their mothers via milk during lactation. For pinnipeds, PBDE transfer from mother to pup was reported to be 44.4 ± 9.9% (n=5) [9]. Thus, it is likely that the difference in PBDE levels for pups in this study was due in large part to the difference in PBDE contamination in their adult mothers. Pre-weaned pups feed one trophic level higher than their mothers, because females remobilize their body tissues to synthesize milk [55]. Harbour and hooded seals occupy the highest trophic levels in the SLME, while Gulf grey and harp seals are at the intermediate trophic level [56]. Harbour seals have the most diverse diet consuming a wide selection of both benthic fish and invertebrates as well as pelagic fish. Grey seals also have a diverse diet but consume greater proportions of demersal and pelagic fish species. Harp seals specialize on schooling pelagic fish species [57]. The main preys include capelin (*Mallotus villosus*) for harp seals; atlantic herring, capelin, and sand lance (*Ammodytes sp.*) for harbour seals; sand lance and atlantic cod (*Gadus morhua*) for grey seals and Greenland halibut (*Reinhardtius villosus*) for hooded seals [57]. Levels of ∑PBDEs in the potential prey of seals are summarized in Table 2. These observations indicate that hooded and harp seals, which presented the lowest ∑PBDEs concentrations in their blubber among the seal species from the SLME, feed on the lowest or intermediate contaminated prey items. On the other hand, many prey species of harbour seal, which is most contaminated SLME phocid, are characterized by high levels of ∑PBDEs (Table 2). Moreover, the very low ∑PBDEs levels observed in harp and hooded seals should also be related to part of the diet of these animals in the Arctic environment.

Results reported in this study indicate that fluvial inputs of PBDE issued from urbanisation explain high PBDE concentrations in serum and blubber of harbour seal pups from SLE. On the other hand, PBDE levels in grey and harp seal pups are in good agreement with the estimated intake of PBDEs of their mothers through their diet in the Gulf of St. Lawrence, which indicate minor PBDE contamination for those species while migrating outside the Gulf of St. Lawrence.

Table 2. Mean concentrations (ng/g lipid weight) of ΣPBDEs in prey of seals from the St. Lawrence Marine Ecosystem (data from [8] and Lebeuf, unpublished data). Fish were sampled in the SLE.

Prey species	sample tissue	lipids (%) (whole body)	ΣPBDEs (ng/g l wt)
Atlantic herring	liver	4	60.7
Greenland halibut	muscle	15.7	19.0
Greenland halibut	liver	15.7	27.9
Atlantic cod	liver	1.4	30.4
Sand lance	whole body	6.5	10.7
Capelin	whole body	1.4	44.8

ACKNOWLEDGEMENTS

Fisheries and Oceans Canada (Maurice Lamontagne Institute, Mont Joli, QC; M.O.H. and M.L.) supported this work. This study was also supported by the Canada Research Chair in Environmental Immunotoxicology (M.F.). Thanks to Samuel Turgeon, Emmanuel Chasson, Harrison MacRae and Wayne Penney for their help with the field work. Thanks to Steve Trottier and Tiphaine Brett provided valuable help in the laboratory.

SUPPORTING INFORMATION AVAILABLE

Tables of species, sampling location, date and characteristics of phocid pups sampled in the Estuary and Gulf of St. Lawrence (Table SI-1) and congener detected (Table SI-2). This material is available free of charge via the internet at http://pubs.acs.org

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SUPPORTING INFORMATION FOR PBDEs IN BLOOD AND BLUBBER OF HARBOUR, GREY AND HARP SEAL PUPS FROM EASTERN CANADA

Age determination

Age as a function of total body weight of unknown-age pups was expressed by the formula: age (days) = (weight-birth weight)/weight gain per day.

- Harp seal. We modified the equation of Stewart and Lavigne [1]: age (days) = 0.35*body weight (kg)-3.2. We used the equation age (days) = [body weight (kg)-10]/2.5
- Grey seal. We used the equation age (days) = [body weight (kg)-16]/2.8 determinated from Bowen et al. [2]
- Harbour seal. We used the equation derived from Dubé et al. [3] : age (days) = [body weight (kg)-11.4]/0.54

Chemical analysis

Seal blubber and serum were analyzed for PBDEs (IUPAC numbers 1, 2, 3, 7, 10, 8/11, 12, 13, 15, 17, 25, 28, 30, 32, 33, 35, 37, 47, 49, 66, 71, 75, 77, 85, 99, 100, 116, 118, 119, 126, 138, 140, 153, 154, 155, 166, 181, 183, 190).

Blubber samples

Each blubber sample was chemically dried with sodium sulphate before being transferred to a glass column. A single 13C12 PCB (PCB-170) was added to the column before the extraction procedure. Lipids and lipophilic compounds were extracted from the sample with dichloromethane-hexane (50:50). The extraction solution received a mixture of three 13C12 PBDEs (PBDE-47, -99 and -153) and was prepared for purification. Lipids were removed from the remaining extract by gel permeation chromatography. The extract was further cleaned by elution through a two-layer column packed with hydrated neutral silica and hydrated alumina. The final extract was reduced in volume and spiked with an instrument performance solution containing two additional 13C12 PCBs (PCB-111 and -189) for a final volume of 100 µL. All solutions were stored in amber glass vials at -20℃.

Quantification of individual PBDEs was performed using either a Varian 3400CX series gas chromatograph equipped with a Varian Saturn TV ion trap, a Varian 1078 split/splitless programmable injector (5 µl injection volume) operated in splitless mode,

and a Varian 8200CX autosampler or a ThermoFinnigan Trace 2000 gas chromatograph equipped with a ThermoFinnigan PolarisQ ion trap, a ThermoFinnigan PTV (programmed temperature vaporizer) split/splitless injector (5 µl injection volume) operated in splitless mode, and a ThermoFinnigan 2000 autosampler. Chromatographic separation of PBDEs was achieved using a 30m DB-5MS column (0.25 mm ID, 0.25 µm film thickness) with helium as the carrier gas. The ion source was operated in electron impact ionisation mode and the ion trap in MS/MS mode.

Concentrations of PBDE congeners were calculated using relative response factors (RRFs) determined from a three-point calibration curve. The accuracy of the method was assessed by measuring the recoveries of known quantities of native congeners added to a blank solution, which subsequently underwent all the analytical steps.

Serum samples

The frozen serum samples were thawed at room temperature. About 2-3 g of precisely weighted serum sample received 50 µl of a surrogate solution containing a mixture of three 13C12-labelled PBDE congeners at a concentration of 200 pg per µL. The serum was denatured with hydrochloric acid (6 M, 1 ml) and isopropanol (3 ml). Hexane:Methyl tert-butyl ether (50:50, 7 ml) was added and the samples were sonicated for 20 min. The phases were separated by centrifugation (189 g, 10 min). The organic phase was transferred and the aqueous phase was extracted two more times. The organic phases were combined and washed with sodium sulftate. The organic phase was transferred to a round-bottom flask and the solvent evaporated. The residues were dissolved in hexane and applied to a pre-washed and conditioned silica solid-phase extraction (SPE) column (Cartouche SPE, J.E. Baker, 500 mg, silica gel SiOH). Elution of the extracts occurred with a total volume of 8 ml of dichloromethane:hexane (15:85). After vaporization of the elution solvent, the extract was spiked with an instrument performance solution containing two additional 13C12 PCBs (PCB-111 and -189) for a final volume of 100 µl. The obtained extract was analyzed by GC/MS/MS as described above.

Table S1. Species, sampling location, date and characteristics of pup phocids sampled in the Estuary and Gulf of St Lawrence.

Species	Sampling location Sector	Region	*n*	Sex	Age (days)	Blubber lip d (%)	Serum lipid (%)	Serum lipid (g/l)	Condition index	Collection date (d/mo/yr)
Harbour seals	Bic	Estuary	6	5M, 1F	5.8 ± 1.4 [a] (1-10)	65 ± 23 (19-79)	1.2 ± 0.4 [ab] (0.9-2)	11.9 ± 4.17[ab] (9-19.9)	67 ± 6 (62-75)	31/05/2007
Harbour seals	Newfoundland	Gulf	6	3M, 3F	6.3 ± 1.2 [a] (3-9)	58 ± 9 (46-70)	0.8 ± 0.2[a] (0.6-1.1)	7.5 ± 2.1[a] (5.5-10.9)	73 ± 8 (63-84)	24,25/05/2008
Grey seals	Amet Island & Hay Island	Gulf	6	3M, 3F	1.5 ± 0.5 [b] (0-4)	64 ± 17 (43-84)	1 ± 0.2 [ab] (0.7-1.3)	10.1 ± 2.2[ab] (6.8-12.7)	75 ± 8 (67-86)	12/01/2008 13/01/2008
Harp seals	Magdalen Islands	Gulf	6	3M, 3F	3 ± 0.8 [ab] (0-6)	72 ± 8 (62-85)	1.5 ± 0.3[b] (1.1-1.9)	14.6 ± 3.2[b] (10.6-19.3)	83 ± 5 (75-90)	6,7/03/2008

Values are given as the mean ± standard error.
Species with significant comparisons within a characteristic (blubber lipid, serum lipid or condition index) are labelled with different letters.

Table S2. Detected sample number for each congener used in article from different seal species (blubber and serum).

Total	Harbour seals Bic *n* = 6	Harbour seals NF *n* = 6	Harp seals *n* = 6	Grey seals *n* = 6
Blubber				
BDE 28	*n* = 5	*n* = 4	*n* = 6	*n* = 3
BDE 33	*n* = 1	*n* = 3	*n* = 1	*n* = 2
BDE 35	*n* = 3	*n* = 1	*n* = 0	*n* = 2
BDE 47	*n* = 5	*n* = 5	*n* = 5	*n* = 5
BDE 49	*n* = 5	*n* = 5	*n* = 3	*n* = 2
BDE 99	*n* = 6	*n* = 6	*n* = 6	*n* = 6
BDE 100	*n* = 6	*n* = 6	*n* = 6	*n* = 6
BDE 126	*n* = 0	*n* = 4	*n* = 0	*n* = 3
BDE 138	*n* = 3	*n* = 4	*n* = 4	*n* = 2
BDE 153	*n* = 6	*n* = 6	*n* = 6	*n* = 6
BDE 154	*n* = 6	*n* = 5	*n* = 5	*n* = 5
BDE 183	*n* = 2	*n* = 2	*n* = 0	*n* = 4
BDE 190	*n* = 1	*n* = 0	*n* = 2	*n* = 1
Serum				
BDE 28	*n* = 0	*n* = 0	*n* = 0	*n* = 0
BDE 47	*n* = 6	*n* = 6	*n* = 6	*n* = 6
BDE 99	*n* = 6	*n* = 3	*n* = 6	*n* = 4
BDE 100	*n* = 6	*n* = 2	*n* = 4	*n* = 4
BDE 153	*n* = 6	*n* = 4	*n* = 2	*n* = 2
BDE 154	*n* = 1	*n* = 3	*n* = 1	*n* = 0

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4.3. Les butylétains dans une chaîne alimentaire de l'Estuaire du Saint-Laurent, Québec, Canada

Article 5 : *Héloïse Frouin, Émilien Pelletier, Véronique Lesage, Michel Lebeuf, Richard St.-Louis, Michel Fournier. Butyltins in a Food Web from St. Lawrence Estuary, Quebec, Canada.* (**Étude préliminaire**)

Mon rôle a consisté à rechercher les données déjà existantes dans la littérature, s'en appuyer pour de nouvelles analyses, interpréter les données et écrire cet article à titre d'auteur principal.

RÉSUMÉ

Le présent article montre que les isotopes stables de l'azote peuvent être utilisés pour estimer le niveau trophique des organismes dans l'Estuaire du St-Laurent. Cette information permet d'étudier le transfert des butylétains à travers une chaîne alimentaire typique du St. Laurent. Par la suite, une base de données plus importante sera utilisée pour étudier différents réseau trophique de l'Estuaire du St. Laurent. Les résultats indiquent une bioamplification de ∑BT, DBT et MBT avec le niveau trophique croissant des organismes dans une chaîne alimentaire typique dans l'Estuaire du St-Laurent.

ABSTRACT

The present article demonstrates than stable isotopes of nitrogen could be used to assess the trophic level of the organisms from St. Lawrence Estuary (SLE). This information give the opportunity to study the butyltin transfer throughout a food chain. In the future, an improved database will use to assess several food web from the SLE. Results indicate a biomagnification of ∑BT, DBT and MBT with increasing trophic level in the food chain.

INTRODUCTION

Tributyltin (TBT) has been extensively used as a biocide in antifouling paints since the 1970s. In 2003, the Marine Environmental Protection Committee (MEPC) recommended a world-wide ban TBT owing to its potential environmental impacts. Despite the bans, butyltins (BTs) are still present in seawater, bottom sediments, and biota in large quantities (Maguire and Batchelor, 2005). Butyltins (BTs), TBT and its major metabolites dibutyltin (DBT) and monobutyltin (MBT), possess both lipophilic and ionic properties that promote bioaccumulation in lipids and binding to macromolecules upon exposure and food chain bioamplification of BTs in the marine environment has been reported (Kannan et al., 1996). Furthermore, the lipophilic nature of TBT induces its adsorption onto organic matter, and its penetration in biological membranes. Plants and algae may take up TBT from seawater, followed by its stepwise transfer to primary consumers and upper predators, leading to its bioaccumulation within the food web.

Trophic transfer of pollutants is the movement of chemicals from one trophic level to the next. Recently, stable isotopes of nitrogen have been used to assess trophic position of the aquatic organisms in food webs and combinated with contaminant levels in organisms, the food web transfer of persistent organic pollutants (POPs) in aquatic food webs could be evaluated. The ratio of the heavier to lighter stable isotopes of nitrogen ($^{15}N/^{14}N$), expressed relative to a standard as $\delta^{15}N$, generally increases with trophic position in aquatic food webs, providing a continuous variable with which to assess both trophic level (Lesage et al. 2001). This information allows to examine food web transfer of POPs (Kiriluk et al. 1995; Fisk et al. 2001; Martin et al. 2004). Stable isotopes of nitrogen provide a number of advantages in assessing trophic position because they are easily determined and incorporate diet over a longer period than stomach contents (De Niro and Epstein 1981). However, this approach shows several considerations or sources of uncertainly that can influence interpretation of data from

field studies (Jardine et al. 2006). Overall food web biomagnification factors (BMFs) can be estimated from slopes of logarithmic concentration of contaminants vs $\delta^{15}N$ values in food web components (e.g. Broman et al. 1992; Fisk et al. 2001).

St. Lawrence River (Eastern Canada) is a major shipping route year-round to eastern harbours and the main entrance to the North American Great Lakes. Extensive data on TBT and its metabolites (Saint-Louis et al. 1997; Saint-Louis et al. 2000; Mamelona and Pelletier 2003; Michaud and Pelletier 2006; Viglino et al. 2006) and determination of the community structure by stable isotope analysis (Lesage et al. 2001) in St. Lawrence Estuary afforded the opportunity to assemble a comprehensive food web to examine trophic transfer of BTs. The objectives of this work were to develop BT-trophic level relationships for a typical food chain from the SLE in order to assess biomagnification of TBT and its metabolites in the SLE.

MATERIAL AND METHODS

Data collections

Ten species were chosen : one benthic macroalgae (*Laminaria longicruris*), four benthic invertebrates (blue mussel, *Mytilus edulis*; green sea urchin, *Strongylocentrotus droebachiensis*; pink shrimp, *Pandalus borealis*; snow crabe, *Chionoecetes opilio*), four benthic fish (golden redfish, *Sebastes marinus*; checker eelpout, *Lycodes vahlii*; american plaice, *Hippoglossoides platessoides*; fourbeard rockling, *Enchelyopus cimbrius*) and one marine mammal (beluga whale, *Delphinapterus leucas*). BT concentrations and stable isotope values for each species were found in the litterature (c.f. Table 1).

Food web TMF and Predator/Prey BAF calculations

Trophic magnification factors (TMFs) were calculated based on the relationship between $\delta^{15}N$, and butyltin contaminant concentrations :

$\ln$ concentration (wet weight) = $a + (b \times \text{trophic level})$ (1)

Relationship between contaminant and trophic level of organisms were analyzed by linear regression (b is the slope and a is the Y-intercept). The calculations were performed using STATISTICA for windows (version 7.0, StatSoft Inc, 1995).

Converting $\delta^{15}N$ to trophic level (TL) was performed according to the relationship:

$TL = 1 + (\delta^{15}N\text{consumer} - \delta^{15}N\text{particulate organic matter})/TEF$ (2)

where $\delta^{15}N$ is expressed as ‰, TEF is the trophic enrichment factor in $\delta^{15}N$ for a particular tissue. This latter value was set to 3.4 ‰ for all community components (Lesage et al. 2001). $\delta^{15}N$ particulate organic matter in the St. Lawrence Estuary was set to 4.5 (Lesage et al. 2001).

A TMF could then be calculated for each contaminant from the slope b in eq. 1 using the formula reported in Martin et al. (2004):

$TMF = e^{b}$ (3)

RESULTS AND DISCUSSION

BT levels in organisms from SLE

Concentrations for lower trophic levels tend to be lower than those of fish or mammals (Table 1), excepted for the pink shrimp, which presented the highest BT levels. A study of organotins in organisms of St. Lawrence Estuary showed total butyltins concentrations in benthic invertebrates ranging from 43.11 (blue mussel) to 311 ng/g d.w. with predominant TBT. For benthic fish, butyltins concentrations varied from 72 (American plaice) to 157 (fourbeard rockling). The BT range of the St. Lawrence Estuary, using ∑BTs, was macroalgae -> blue mussel -> snow crab -> green sea urchin -> american plaice -> checker eelpout -> golden redfish -> fourbeard rockling -> pink shrimp -> beluga (male and female).

Trophic levels of organisms

The trophic status of the St. Lawrence Estuary, using nitrogen isotope, was macroalgae, blue mussel, green sea urchin -> pink shrimp, snow crab (decapods) -> golden redfish, checker eelpout, american plaice, fourbeard rockling (fish) -> beluga (top predator). The $\delta^{15}N$ values range from 7.36 (blue mussel) to 16.31 (male beluga whale) (Table 1).

Benthic fish are closely related to sediment fauna because of their feeding habits. Most of the fish used in the present study are carnivorous and feed on crustaceans, molluscs, worms and small fish (Scott and Scott 1988). According to Takahashi et al. (1997), predators such as carnivorous fish used in our study, can contain high concentrations of butyltins in their tissues even if they often have a high metabolic capacity (Lee 1985). Food is suspected as the main contribution for bioaccumulation in

St. Lawrence fish as a weak biomagnifications towards carnivorous benthic fish is observed.

The use of stable isotope analysis in studies of animal ecology rests on two central tenets : 1) stable isotope ratios in consumer are proportional to those in their putative diet, and 2) differences in isotope ratios exist among food sources available for consumers. The stable isotope approach provides diet estimates that are not affected by the differential rate of disappareance of prey in stomach contents or faeces. It bypasses the problems associated with the high frequency of empty stomachs found in marine mammals and estimates the assimilated, not just ingested, food. However, one drawback to the stable isotope approach is that it cannot identify which species from a given trophic level were consumed. Creating models that estimate the relative contribution of different prey to the diet of a consumer requires some basic knowledge of potential prey species. However, this approach provides a reliable picture of the contribution of invertebrates to the diet and indicates that beluga whale may derive a considerable amount of its energy from invertebrate food ressources.

Food web

A general scheme of food web (Fig. 1) has been prepared using mean concentrations of ∑BTs calculated for each species (combining all sampling locations and all sampling years). There is no evidence that the organisms considered in this article represent a real food web, however concentrations of ∑BTs increased with the trophic level. Thus, although, this food web is fictive, we assume that a relationship exists between BT accumulation and level trophic of the organisms (i.e. the biomagnification).

In the present situation, dietary uptake is assumed to be the main entrance for pollutants in beluga tissues. Diet and feeding modes could be factors influencing the difference of accumulation of butyltin compounds between males and females in beluga whales. Females with their calves are found mainly in the southern part of the Estuary during the summer, whereas other adults prefer to live in the St. Lawrence Trough (Michaud, 1993). Secondly, beluga whales can consume small fish and invertebrates. Previous studies demonstrated that content from 2 stomachs collected in 1989 and 1995, and diet reports from the late 1930s, indicated that invertebrates such as *Nereis* sp., worms and shrimp-like decapods, as well as Atlantic tomcod (*Microgadus tomcod*), sand lance (*Ammodytes* sp.) and redfish (*Sebastes* sp.) may be important species in the

St. Lawrence beluga's diet (Vladykov, 1956; Béland, 1995; Lesage and Hammill unpubl. data). Moreover, a particular feeding technique used by beluga is the violent expulsion of water from the mouth to release invertebrates from the sea bottom (Fontaine 2005). Thus, as is rarely the case amongst toothed whales, they feed heavily on benthic organisms. Considering the known accumulation of TBT and its breakdown products in sediments, the consumption of contaminated benthic organisms could represent a notable pollutant pathway.

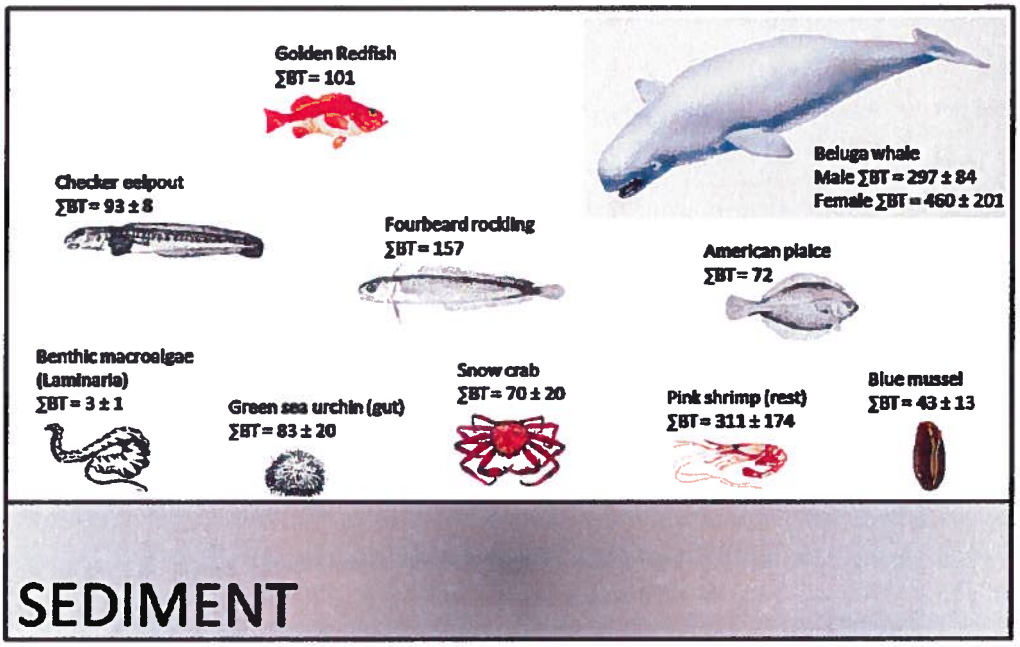

Figure 1. Mean ∑BTs concentrations (ng/g wet weight) in specific tissues or whole organisms of St. Lawrence Estuary.

Table 1 : Species, year of sampling, BT concentrations and stable isotopes values of organisms from SLE

Organism (tissue)	BT concentrations				Year of sampling	Reference	Stable isotope values		Year of sampling	Reference
	MBT	DBT	TBT	∑BTs			$\delta^{15}N$	$\delta^{13}C$		
Benthic macroalgae	2.81 ± 1.14	0	0.4 ± 0.08	3.21 ± 1.08	2002	Mamelona and Pelletier (2003)	7.90 ± 0.04	-13.83 ± 0.02	1996	Lesage et al. (2001)
Pink shrimp (rest)	46 ± 31	28 ± 11	266 ± 130	311 ± 174	2000-2002	Viglino et al. (2006)	13.16 ± 0.18	-17.81 ± 0.04	1995	Lesage et al. (2001)
Snow crab	8 ± 4.64	13 ± 10.41	48.75 ± 24.48	70.25 ± 20.36	2002	Viglino et al. (2006)	13.27 ± 0.28	-17.71 ± 0.30	1995-1996	Lesage et al. (2001)
Blue mussel	0.96 ± 0.96	23.03 ± 7.09	19.12 ± 6.68	43.11 ± 12.81	1995	Saint-Louis et al. (1997)	7.36	-19.23	1996	Lesage et al. (2001)
Green sea urchin (gut)	24.90 ± 3.82	5.75 ± 1.07	52.74 ± 16.65	83.39 ± 19.83	2002	Mamelona and Pelletier (2003)	7.75 ± 0.38	-14.61 ± 0.10	1996	Lesage et al. (2001)
Golden redfish				101	2003	Michaud and Pelletier (2006)	13.87 ± 0.13	-19.06 ± 0.13	1996	Lesage et al. (2001)
Checker eelpout	21 ± 8	27 ± 1	46 ± 16	93 ± 8	2000-2001	Viglino et al. (2006)	14.62 ± 0.56	-17.94 ± 0.45	1995-1996	Lesage et al. (2001)
American plaice				72	2003	Michaud and Pelletier (2006)	14.72 ± 0.19	-18.13 ± 0.09	1996	Lesage et al. (2001)
Fourbeard rockling				157	2003	Michaud and Pelletier (2006)	15.64	-18.80	1996	Lesage et al. (2001)
Male Beluga whale (blubber)	45.91 ± 35.39	207.36 ± 59.25	44.45 ± 14.58	297.27 ± 83.67	1995-1998	Saint-Louis et al. (2000)	16.31 ± 0.56	-16.48 ± 0.13	1997	Lesage et al. (2001)
Female Beluga whale (blubber)	12.4 ± 2.08	351 ± 153.43	97.7 ± 50.26	460.6 ± 201	1995-1998	Saint-Louis et al. (2000)	15.40 ± 0.18	-17.01 ± 0.10	1997	Lesage et al. (2001)

Relationship between BTs and trophic level

For an equivalent trophic level, BTs are clearly located in the rest (head and cuticle) of pink shrimp (∑BTs = 311 ng/g) whereas snow crab (∑BTs = 70 ng/g) experienced much lower levels in its tissues. ∑BT, TBT, DBT and MBT data generated an association (expressed with TMF) between concentration and trophic level among all organisms (Fig. 2). This is justified because all these species are benthic and beluga whale could have a benthic diet rather than pelagic diet. For ∑BT, DBT and MBT statistically significant trends were observed whereby concentrations increased with increasing trophic level, suggesting biomagnifications in the benthic food web. TBT data did not generate a significant association between concentration and trophic level among all organisms, although TMF was higher than 1 (i.e. TMF = 2.63). Thus, the ∑BT biomagnification could more result from DBT and MBT than from TBT. Biomagnification of organotins through food chains also has been found in high trophic aquatic organisms, such as myctophid fish (Takahashi et al. 2000), marine mammals and fish-eating birds (Kannan et al. 1996; Stäb et al. 1996; Kannan and Falandysz 1997; Strand and Jacobsen 2005).

Because of the importance of the diet as a route of exposure for severall persistent contaminants, stable isotope analysis has considerably advanced the field of ecotoxicology by linking wild animal populations to their diet and ultimate contaminant source. However, stable isotope analysis presents several considerations or sources of uncertainty (resumed in Jardine et al. 2006). For example, different tissues from an individual animal have variable stable isotope ratios. Because different tissues may have different degrees of fractionation relative to the diet and turnover rates, a single tissue may not adequate in tracing the flow of organic matter and contaminant through an ecosystem. More complete analysis is required to examine the question further (tests with different tissues from an organism).

The present article demonstrates that butyltins are bioamplified within aquatic food web of St. Lawrence Estuary.

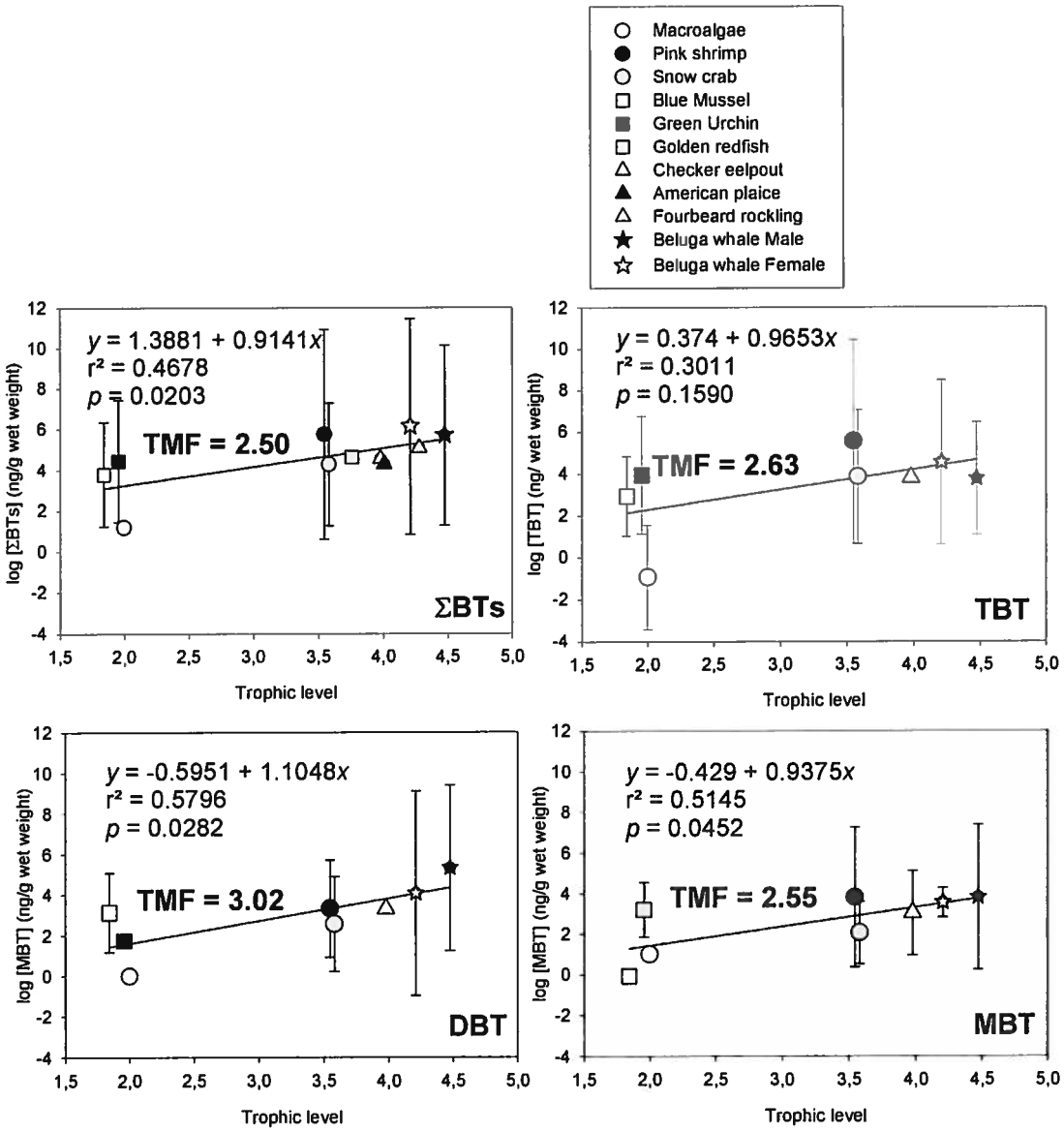

Figure 2. Plots of mean BT concentration (ng/g ww ± standard error) in organisms from St. Lawrence Estuary versus trophic level. Linear regression of log data revealed significant biomagnification (i.e. TMF >1).

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5. TOXICOLOGIE

5.1 Effets toxiques *in vitro* du tributylétain et de ses metabolites sur les cellules immunitaires du phoque commun (*Phoca vitulina*)

Article 6: *Frouin H., Lebeuf M., Saint-Louis R., Hammill M., Pelletier É., Fournier M. 2008. Toxic effects of tributyltin and its metabolites on harbour seal (Phoca vitulina) immune cells in vitro. Aquatic Toxicology 90, 243-251.*

Mon rôle a consisté à faire l'échantillonnage, les analyses, interpréter les données et écrire cet article à titre d'auteur principal.

RÉSUMÉ

La contamination environnementale par les butylétains, leur bioaccumulation et leurs effets comme perturbateurs endocriniens dans la vie sauvage sont bien documentés. Bien que suspectés, les effets potentiels de l'exposition aux butylétains sur le système immunitaire des mammifères marins ont été peu étudiés. Dans cette étude, nous avons determiné les effets du tributylétain et de ses produits de dégradations, le dibutylétain (DBT) et le monobutylétain (MBT) sur les réponses immunitaires de phoques commun. Les cellules mononuclées du sang périphérique ont été isolées de phoques communs nouveau-nés et d'adultes et exposées *in vitro* à des concentrations croissantes de butylétains. Le DBT induit une diminution significative de l'activité phagocytaire à 100 nM et à 200 nM et réduit significativement l'efficacité phagocytaire à 200 nM chez les adultes. Aucun effet sur la phagocytose n'a été observé avec le TBT et le MBT. Chez les nouveau-nés, la plus haute concentration de DBT (200 nM) inhibe l'efficacité phagocytaire. Une réduction de la capacité de destruction des tumeurs des cellules *Natural killer* (NK) chez les adultes se produit quand les leucocytes sont incubés *in vitro* avec 50 nM de DBT et 200 nM de TBT durant 24 h. Chez les phoques adultes, la proliferation des lymphocytes T est significativement supprimé quand les cellules sont exposées à 200 nM de TBT et 100 nM de DBT. Chez les nouveau-nés, la réponse proliférative augmente après une exposition à 100 nM de TBT et 50 nM de DBT, mais diminue avec 200 nM de TBT et 100 nM de DBT. Les fonctions immunitaires sont plus affectées par l'exposition aux butylétains chez les adultes que chez les nouveau-nés, suggérant que d'autres mécanismes non suspectés pourraient affecter les paramètres immunitaires chez les nouveau-nés. Le potentiel toxique des butylétains suit l'ordre: DBT > TBT > MBT. Les concentrations de butylétains chez les phoques commun nouveau-nés de l'estuaire du

St.-Laurent (Parc National du Bic) vont de 0,1 à 0,4 ng Sn/g de poids humide et 1,.2-13,4 ng Sn/g de poids humide dans le sang et le gras, respectivement. Pour ces animaux, les concentrations de DBT se trouvent constamment sous la limite de quantification de 0,04 ng Sn/g de poids humide dans le sang et de 0,2 ng Sn/g de poids humide dans le gras. Les resultants suggèrent que les concentrations mesurées dans les nouveau-nés sont trop faibles pour induire des effets toxiques sur leur système immunitaire durant les premiers jours de leurs vies. Cependant, sur la base de nos résultats *in vitro*, nous proposons que les butylétains et le DBT en particulier, pourraient poser une menace sérieuse aux fonctions immunitaires des phoque commun adultes.

Aquatic Toxicology 90 (2008) 243–251

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Toxic effects of tributyltin and its metabolites on harbour seal (*Phoca vitulina*) immune cells *in vitro*

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ARTICLE INFO

Article history:
Received 10 June 2008
Received in revised form 1 September 2008
Accepted 5 September 2008

Keywords:
Butyltins
Immunotoxicity
Harbour seals
Lymphocytes
NK cells
Phagocytosis

ABSTRACT

The widespread environmental contamination, bioaccumulation and endocrine disruptor effects of butyltins (BTs) to wildlife are well documented. Although suspected, potential effects of BTs exposure on the immune system of marine mammals have been little investigated. In this study, we assessed the effects of tributyltin (TBT) and its dealkylated metabolites dibutyltin (DBT) and monobutyltin (MBT) on the immune responses of harbour seals. Peripheral blood mononuclear cells isolated from pup and adult harbour seals were exposed *in vitro* to varying concentrations of BTs. DBT resulted in a significant decrease at 100 and 200 nM of phagocytotic activity and reduced significantly phagocytic efficiency at 200 nM in adult seals. There was no effect in phagocytosis with TBT and MBT. In pups, the highest concentration (200 nM) of DBT inhibited phagocytic efficiency. A reduction of tumor-killing capacity of adult *natural killer* (NK) cells occurred when leukocytes were incubated *in vitro* with 50 nM DBT and 200 nM TBT for 24 h. In adult seals, T-lymphocyte proliferation was significantly suppressed when the cells were exposed to 200 nM TBT and 100 nM DBT. In pups, the proliferative response increased after an exposure to 100 nM TBT and 50 nM DBT, but decreased with 200 nM TBT and 100 nM DBT. The immune functions were more affected by BTs exposure in adults than in pups, suggesting that other unsuspected mechanisms could trigger immune parameters in pups. The toxic potential of BTs followed the order of DBT > TBT > MBT. BT concentrations of harbour seal pups from the St. Lawrence Estuary (Bic National Park) ranged between 0.1–0.4 ng Sn/g wet weight (ww) and 1.2–13.4 ng Sn/g ww in blood and blubber, respectively. For these animals, DBT concentrations were consistently below the quantification limit of 0.04 ng Sn/g ww in blood and 0.2 ng Sn/g ww in blubber. Results suggest that concentrations measured in pups are considered too low to induce toxic effects to their immune system during first days of life. However, based on our *in vitro* results, we hypothesize that BTs, and DBT in particular, could pose a serious threat to the immune functions in free-ranging harbour seal adults.

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1. Introduction

The oceans are the ultimate sink in the biogeochemical cycles of several xenobiotics. Many of them are immunosuppressive (De Swart et al., 1996; Lahvis et al., 1995) and are thought to have contributed to the high mortality observed in several marine mammalian species during recent morbillivirus epizootics (Osterhaus et al., 1995; Ross et al., 1996). Indeed, nearly 20,000 harbour seals (*Phoca vitulina*) and several hundred grey seals (*Halichoerus grypus*) were found dead in the North Sea in 1988 (Dietz et al., 1989). Similarly, 2500 bottlenose dolphins (*Tursiops truncatus*) were found dead in the US east coast waters (Kuehl et al., 1991) and 8000 Baikal seals (*Phoca sibirica*) died in Lake Baikal in 1987–1988 (Grachev et al., 1989). A more detailed study of these mortalities revealed three common features. First, all dead animals had a severe viral infection. Second, most epizootics occurred near industrial regions or in semi-closed ecosystems. Third, elevated concentrations of persistent toxic contaminants were found in the organs of all affected animals, suggesting that contaminants found in the marine environment may represent a risk to the health of free-ranging marine mammals. The degree of impact on wild populations, however, remains uncertain.

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doi:10.1016/j.aquatox.2008.09.005

Table 1
Details of harbour seal (*Phoca vitulina*) samples analysed in this study.

Date of collection (2007)	Location (Canada)	*n*	Sex[a]	Age	Tissues analysed
April	Aquarium (Shippagan)	4	M:3, F:1	Adult	Blood
May	Zoo (Saint-Félicien)	4	F:4	Adult	Blood
May	Bic National Park	6	M:5, F:1	Pups	Blubber, blood

[a] M (male); F (female).

Tributyltin (TBT) has been extensively used as a biocide in antifouling paints since the 1970s. In 2003, the Marine Environmental Protection Committee (MEPC) recommended a world-wide recommendation ban TBT owing to its potential environmental impacts. Despite the bans, butyltins (BTs) are still present in seawater, bottom sediments, and biota in large quantities (Maguire and Batchelor, 2005). BTs possess both lipophilic and ionic properties that promote bioaccumulation in lipids and binding to macromolecules upon exposure and food chain bioamplification of butyltins in the marine environment has been reported (Kannan et al., 1996).

TBT is reported to cause serious health problems, such as disrupting the endocrine system of low trophic level organisms in the aquatic food chain and is suspected to have immunotoxic effects on marine organisms (Matthiessen and Gibbs, 1998; Kannan et al., 1998). The toxicity of organotin compounds has been demonstrated in laboratory and marine animals and in humans (Antizar-Ladislao, 2008). In experimental studies, BTs showed strong toxicity on cells of the immune system, manifested by thymus atrophy, reduction in spleen weight and cytotoxicity to bone marrow and red blood cells at relatively low exposure concentrations (Boyer, 1989). TBT and DBT have been shown to decrease thymocyte proliferation (Seinen and Penninks, 1979). TBT has also been reported to cause apoptosis in peripheral blood lymphocytes (Stridh et al., 2001) and affect the function of natural killer (NK) lymphocytes (Whalen et al., 1999). Because BTs concentrations in the liver of beluga whales from St. Lawrence Estuary were reported to range between 40 and 2100 ng Sn/g wet weight (ww) (St-Louis et al., 2000), it is suggested that immunosuppression might have occurred in marine mammals living in this area.

In pinnipeds found dead along the coasts of California, USA, noticeable levels of BTs were detected in tissues of diseased animals (Kajiwara et al., 2001), which were suspected to be linked to immunosuppression (Kannan et al., 1997). However, these concentrations are less than those reported in cetaceans and mustelids from coastal waters of the United States (Kannan et al., 1997, 1998). Lesser concentrations of butyltins in pinnipeds have been explained by the excretion of butyltins via molting and effective metabolism (Kim et al., 1996). Because TBT passes through the placental barrier (Iwai et al., 1982; St-Louis et al., 2000) and transfers to milk (Kimura et al., 2005), the effects of TBT exposure on the next generation are of particular concern.

The harbour seal (*Phoca vitulina*) is an ideal model organism for investigating contaminant-induced immune alterations in marine mammals (Ross, 2000). Seals eat contaminated fish, are long-lived, maintain large adipose deposits, and occupy high trophic levels in the marine food chain; they thus accumulate relatively high levels of lipophilic contaminants (Ross et al., 1996). Harbour seals are of interest because they are non-migratory and they inhabit a variety of habitats including coastal areas that are both uncontaminated and contaminated.

The objectives of this work were to investigate whether tributyltin, dibutyltin and monobutyltin exert any *in vitro* effects on the immune system of harbour seal and to determine the current status of the contamination by butyltin compounds in harbour seal pups from a free-ranging population of the St. Lawrence Estuary.

2. Materials and methods

2.1. Animal capture, handling, and sample collection

Six pup harbour seals *Phoca vitulina* L. were collected during May 2007 in Bic National Park near "Bic Island" (48°24′N, 68°51′W), along the south shore of the St. Lawrence Estuary in Quebec, Canada (details in Table 1). Seals were captured in the water using a dip net and an inflatable boat, and subsequently transferred to a larger boat where all handling took place. Prior to analysis, seals were weighed (to ±0.5 kg), tagged, and their sex noted (Dubé et al., 2003). All handling of the pup was carried out as quickly as possible (within 20 min) to avoid abandonment of the pup by the mother (Boulva and McLaren, 1979). Pups were captured throughout the lactation period. Seals were aged by appearance and mass. The blood samples, up to 50 ml, were taken from the extradural intravertebral vein into Vacutainer tubes (Becton-Dickinson, NJ, USA) and were immediately stored at 4 °C until the time of analysis (6 h after sampling). The volume of blood collected was no more than 10% of the total blood volume (McGuill and Rowan, 1989). A biopsy was taken through the skin into the subcutaneous blubber layer using a punch. All research was approved by the Animal Care Committees of the Department of Fisheries and Oceans.

Fresh blood samples were also collected from eight harbour seal adults housed in aquariums (Centre Marin de Shippagan, Canada and Zoo de Saint-Félicien, Canada) (details in Table 1). These samples were taken from a superficial plantar flipper veins and approximately 10–40 ml of whole bloods were collected from each animal. Blood was kept at 4 °C until the time of analysis, 6–8 h after sampling.

2.2. Chemical analyses

Butyltin compounds including TBT and its metabolites DBT and MBT were analysed in selected tissues of six harbour seal pups by gas chromatography as their volatile ethylated derivatives. The extraction procedure used for the butyltin analysis was adapted from Békri and Pelletier (2004). Approximately 1 g of blood or 0.2 g of blubber was placed in a Teflon tube (40 ml) with 5 g of tetramethyl ammonium hydroxide (TMAH, 25% water, Sigma–Aldrich). The tubes were placed in an ultrasonic bath for 60 min at 60 °C. After the addition of 25 ml acetate buffer 1 M at pH 4.1, 4 ml hexane:toluene (1:1), 2 ml NaCl saturated water and 0.6 ml sodium tetraethylborate 2% in water, the tubes were mechanically shaken for 30 min on a wrist shaker. To facilitate phase separation, the tubes were centrifuged (1000 × *g*) for 10 min. The organic phase was collected in a glass test tube with a Teflon cap and a second extraction was performed on the aqueous phase. The combined organic extracts were cleaned up in a first step by adding directly to the tube 1 g of Florisil, then hand shaking and centrifuging (1000 × *g* for 10 min). The organic phase was recovered and cleaned up a second time by passing it through a silica gel/sodium sulfate column made with a Pasteur pipette and eluted with hexane:toluene in a precision pyrex tube. After the addition of tetrapropyltin as the internal standard, the volume of the extract was concen-

trated to approximately 200 μl under a gentle stream of nitrogen at room temperature and transferred to a glass vial with a glass liner (200 μl) and a Teflon cap. Chromatographic separation and quantification were performed on Trace GC coupled to a Polaris Q ion trap mass spectrometer (ThermoFinnigan) equipped with a DB5-MS capillary column (30 m, 0.25 mm i.d., J&W Scientific). Helium was used as the carrier gas at a flow rate of 1.0 cm^3 min^{-1}. Quantitative analysis was based on five-point calibration curves with concentrations (as tin atom) ranging from 4 to 140 ng ml^{-1}; with tetrapropyltin as an internal standard at a concentration of 40 ng ml^{-1}. Based on the lowest concentration of the calibration curve and the final volume of 200 μl, the quantification limits for MBT, DBT and TBT, in blubber, were estimated to be 1.2, 0.2 and 0.7 ng Sn/g ww, respectively. In blood, the quantification limits for MBT, DBT and TBT were 0.18, 0.04 and 0.10 ng Sn/g ww, respectively. The recovery efficiency of the method was determined from the analysis of the certified reference material CRM 477 (mussel tissue, from the National Institute of Standard and Technology) and was estimated to 80% for MBT, 60% for DBT and 75% for TBT.

2.3. Isolation of peripheral blood leukocytes (PBL)

The blood was diluted 1:1 in phosphate buffer saline (PBS) then the cellular suspension was carefully laid on a lympholyte-mammal density solution (1.086 g/ml; Cedarlane Laboratories Ltd., Hornby, Canada). The leukocytes were then isolated by centrifugation for 20 min at 800 × g. The PBL layer was washed three times with PBS at 800 × g and resuspended in RPMI-1640 (Sigma) supplemented with 10% heat inactivated foetal bovine serum (FBS), 1% Pen-Strept and 10 mM Hepes. The ratio of live/dead cells was assessed using trypan blue dye exclusion and visual examination under a fluorescence microscope with a hemacytometer. Cell viability was always greater than 90%. The cells were kept overnight at 4 °C.

This method of isolation was originally designed to isolate viable lymphocytes. However, scattergram of the flow cytometric profiles of the cell suspension (Fig. 1) indicates that all leukocytes subpopulations (i.e., lymphocytes, granulocytes and monocytes) were present in our cell suspensions.

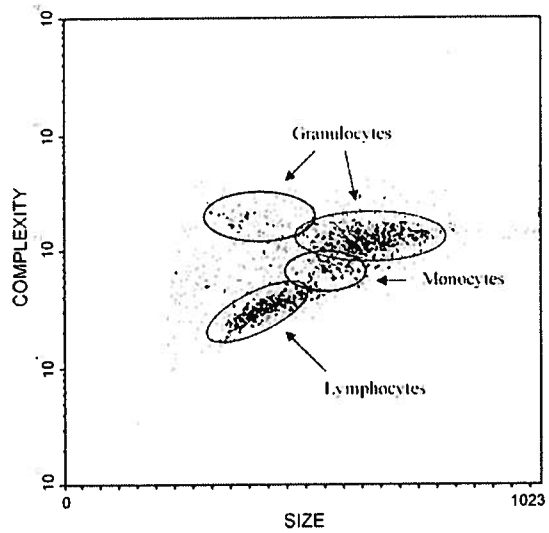

Fig. 1. Scattergram of the flow cytometric profile of PBL isolated as described under Section 2. In order to identify different cell subpopulations on the basis of their size and complexity while at the same time excluding cellular debris, the flow cytometric method was used in the present study. Three populations were isolated by analytical gates, granulocytes, monocytes and lymphocytes.

2.4. Chemical preparation

Butyltin trichloride (TBT), dibutyltin dichloride (DBT) and tributyltin chloride (MBT) were obtained from Aldrich Chemical Co. Dimethylsulfoxide (DMSO) was purchased from Sigma Chemical Co. MBT and TBT were liquid and used as such. DBT was a neat standard, dissolved in DMSO. Concentrations of TBT, DBT and MBT were adjusted by the addition of a stock solution (10^{-2} M of BT^+) of butyltin dissolved in DMSO. This always represented less than 10% of the total volume of the cell suspension. The final concentration of DMSO did not exceed 0.005% (TBT and DBT) and 0.0005% (MBT). Equivalent volumes of DMSO were added to control cells.

2.5. In vitro exposures

For viability, phagocytosis and cytotoxic activity of natural killer (NK) cells, cells adjusted to a concentration of 10^6 cells/ml were exposed at 37 °C in a humidified 5% CO_2 atmosphere for up to 24 h to 25, 50, 100 and 200 nM of each butyltin in gelatin media (0.5% gelatin replaced FBS in complete medium). The cytotoxicity of TBT studied *in vitro* was greatly influenced by serum (FBS or serum protein) in the cell culture medium. Cells were therefore incubated with serum-free medium to reveal an exact threshold concentration of TBT to exert the cytotoxic action during a short period of incubation (Umebayashi et al., 2004). Gelatin was used as an alternative to FBS in complete medium in order to avoid any possible binding of the BTs by serum albumin, which could interfere with delivery of the compounds to the cells.

For lymphocyte proliferation, cells adjusted to 2.5 × 10^6 cells/ml were exposed at 37 °C in a humidified 5% CO_2 atmosphere for up to 66 h to 5, 10, 50, 100, 200 and 500 nM of butyltin in complete medium (RPMI supplemented with 10% FBS, 1% Pen-Strept and 10 mM Hepes). This medium was chosen after preliminary tests had shown that the cell viability of lymphocytes incubated in FBS-free medium was strongly decreased by up to 30% when the incubation time was longer than 24 h. Even in the presence of 10% FBS, the prolonged incubation with nanomolar concentrations of BTs exerted a powerful inhibitory action on lymphocytes.

2.6. Viability

Viability of granulocytes and lymphocytes after exposure was evaluated by flow cytometry using propidium iodide (PI) (100 μg/ml) as described by Brousseau et al. (1999). PI enters in all cells but is actively excreted only by living cells. Samples were analysed with a FACScalibur flow cytometer (Becton Dickinson, San José, USA) and the fluorescence of 5000 events was read in FL3. Data are expressed as a percent of viable cells.

2.7. Phagocytosis

Following the 24 h incubation, phagocytic activity was measured in 8 adults and 6 pups, based on the protocol of Brousseau et al. (1999). We used fluorescent latex beads that were ingested by cells. Samples were analysed with a FACScalibur flow cytometer (Becton Dickinson, San José, USA) equipped with a 488-nm argon laser. For each sample, the fluorescence of 10,000 events was recorded. Results were analysed with the Cell Quest Pro software (Becton Dickinson) to determine the percentage of PBLs that engulfed one bead and more (phagocytic activity) or three beads and more (phagocytic efficiency). The absence of fluorescent response observed in lymphocytes corresponds to their inability to perform phagocytosis and represents an appropriate negative control. The results were expressed as the percentage of phagocytosis.

2.8. Determination of NK activity

To determine the possible effects of the TBT and DBT on NK cell activity, peripheral blood NK cell-mediated lysis of murine lymphoma cells (YAC-1 cells) was determined by a flow cytometry method (Brousseau et al., 1999). Due to limited sample volumes, only 3 adult female seals were included in the experiment. Data were acquired by FACScalibur (Becton Dickinson) flow cytometer with a threshold set at FL1 (DiO+ cells) to exclude effector cells and bare cell nuclei that were DiO negative. A total number of 10,000 events were collected. Data were analysed by the CellQuest Pro software (Becton Dickinson) to determine target cell death (cytolethality). When an NK cell damaged the membrane of a target cell, PI could no longer be excluded and the target cell was stained by PI to become DiO+, PI+. In contrast, target cells not affected by NK cells excluded PI to remain DiO+, PI−. To combine results from separate experiments (using cells from different seals) the levels of lysis were normalized as the percentage of the lysis of the control cells in a given experiment. The results are expressed in percentage lysis of control cells.

2.9. Determination of mitogen-induced lymphocyte blastogenesis

The ability to undergo blastogenesis was measured by ^{3}H-thymidine uptake in 8 adults and 6 pups. Briefly, PBLs (100 μl) were plated with 100 μl mitogen and 10 μl of BTs for 48 h at 37 °C and 5% CO_2 in 96-well round-bottom plates. The optimal concentration used to stimulate T lymphocytes was 5 μg/ml concanavalin A (con-A) (Mori et al., 2005). Samples were tested in triplicate. After a 48-h incubation, 1 μCi of ^{3}H-thymidine was added in each well and plates were incubated for a further 18 h. The cells were harvested on fibreglass filter with a semi-automatic cell harvester and the amount of radioactivity incorporated was measured with a Beckman β-scintillation counter. The raw data were expressed as counts per minute (CPM), triplicates were averaged, and the results were presented as the percentage of the control.

2.10. Statistical analysis

All data are presented as the mean ± standard error. The results were statistically analysed using SigmaStat (Release 3.0) (Jandel, Scientific Software, San Rafael, CA). Normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene–Median test) were first checked and a parametric (ANOVA) or a non-parametric (Kruskall–Wallis) one-way analysis of variance was subsequently performed on the data. The error α was fixed at $P < 0.05$ for all tests.

For the proliferation assay, triplicates were averaged, and the mean and standard deviation were determined. A one-way analysis of variance (ANOVA) with Dunn's method was used to compare the different experimental groups to the control, using $P \leq 0.05$ for statistical significant.

Dose values for inhibition of 50% of proliferation response (ID50) by BTs were obtained statistically by linear regression.

3. Results

3.1. Chemical analysis

Concentrations of MBT, DBT, and TBT blood and blubber of seal pups ranged from below the quantification limits up to 11.2 ng Sn/g ww (Table 2). The blood compartment possessed some lower concentrations of BTs (from 0.1 to 0.4 ng Sn/g ww) than blubber (from 1.2 to 13.4 ng Sn/g ww) in the same animal. TBT was detected in 4 blood samples (from 0.1 to 0.4 ng Sn/g ww) and MBT was detected in 2 blood samples (0.1 and 0.4 ng Sn/g ww). TBT was detected in 4 blubber samples (from 6.3 to 11.2 ng Sn/g ww) and MBT was detected in 3 blubber samples (from 1.2 to 2.5 ng Sn/g ww). The levels of DBT in both blood and blubber of pups were too low to be detected. There was no correlation between blood and blubber concentrations.

Table 2
Concentrations (ng Sn/g ww) in blood and blubber of harbour seal pups from Bic National Park.

Tissue	Sample no.	Sex	MBT	DBT	TBT	ΣBTs[a]
Blood	Pup 1	M	0.1	<0.04	0.1	0.2
Blood	Pup 2	M	0.4	<0.04	<0.1	0.4
Blood	Pup 3	F	<0.18	<0.04	<0.1	<0.18
Blood	Pup 4	M	<0.18	<0.04	0.4	0.4
Blood	Pup 5	M	<0.18	<0.04	0.4	0.4
Blood	Pup 6	M	<0.18	<0.04	0.1	0.1
Blubber	Pup 1	M	2.5	<0.2	6.3	8.8
Blubber	Pup 2	M	2.3	<0.2	11.2	13.5
Blubber	Pup 3	F	1.2	<0.2	<0.7	1.2
Blubber	Pup 4	M	<1.2	<0.2	7.0	7.0
Blubber	Pup 5	M	<1.2	<0.2	8.8	8.8
Blubber	Pup 6	M	<1.2	<0.2	<0.7	<1.2

[a] ΣBTs = TBT + DBT + MBT.

3.2. Viability

The percentage of viable cells was determined by flow cytometry after *in vitro* exposure to BTs. There were no statistically significant treatment-related effects on the viability of immune cells (data not shown).

3.3. Effects on phagocytic activity in adults and in pups following exposure to butyltins

There were no obvious effects of TBT on cells from adult seals (Figs. 2 and 3). At the lowest DBT concentrations (25 and 50 nM) there was no significant effect on phagocytic activity of adult seal PBLs (Fig. 2). In adults, exposure to the highest DBT concentrations (100 and 200 nM) reduced significantly phagocytic activity of cells compared to unexposed cells. At 200 nM DBT a significant reduction on phagocytic efficiency was observed (Fig. 3). In pups, there was no effect of TBT on phagocytic activity or on phagocytotic efficiency (Figs. 2 and 3). At the highest DBT concentration (200 nM) a significant inhibition of phagocytic efficiency occurred in pups. The exposure to MBT had no effect on phagocytic activity in both adults and pups. Phagocytic activity was significantly greater in pups compared to adults (Fig. 2).

3.4. Cytotoxic function of NK cells exposed to butyltins

The patterns of inhibition on NK lytic function (Fig. 4) were similar between the 40:1 and 20:1 ratios. Variations in the ability of NK cells to lyse tumor cells after exposure to TBT showed a significant decrease with 100 and 200 nM for the 20:1 ratio (NK:target) and with the highest concentration at the 40:1 ratio. At the 40:1 and 20:1 ratios, DBT caused a statistically significant decrease in NK function at 50, 100 and 200 nM.

3.5. Tributyltin and dibutyltin decrease lymphocyte proliferation of seal PBLs

The thymidine incorporation assay was used to monitor the proliferative activity of harbour seal PBLs in the presence or absence of butyltin compounds (Fig. 5). In adults, a significant decrease in

the percentage of lymphocyte proliferation was found at concentrations of 200 nM of TBT and 100 nM of DBT. In pups, after stimulation by the mitogen, exposure to 200 nM for TBT increased significantly the proliferative response and was significantly reduced at the highest concentration of TBT (500 nM) and at 100 nM of DBT.

3.6. ID50

Dose values for inhibition of 50% of the proliferation response (ID50) (Table 3) indicate that no TBT values for pups and no MBT values for adults and pups could be calculated because the proliferation response did not decrease below 50% for the range of tested concentrations. The ID50 of DBT showed a similar value for the pups and the adults. In adults, the ID50 of TBT was two orders of magnitude higher compared to that of DBT. In males, the ID50 value for DBT was slightly higher and value for TBT was clearly higher than in females.

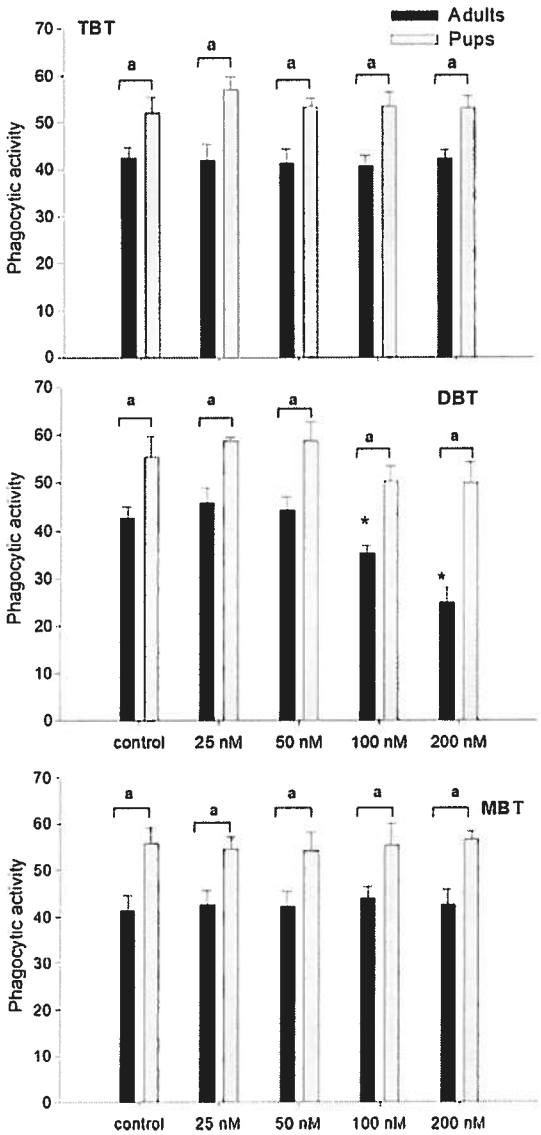

Fig. 2. Effects of butyltins, TBT, DBT and MBT on the activity of phagocytosis. Values are calculated as the percentage of macrophages having engulfed one bead or more. Results are given as mean ± standard error. Asterisk shows a statistical difference from control cells ($P < 0.05$). Letter indicates significant differences between adults and pups ($P < 0.05$).

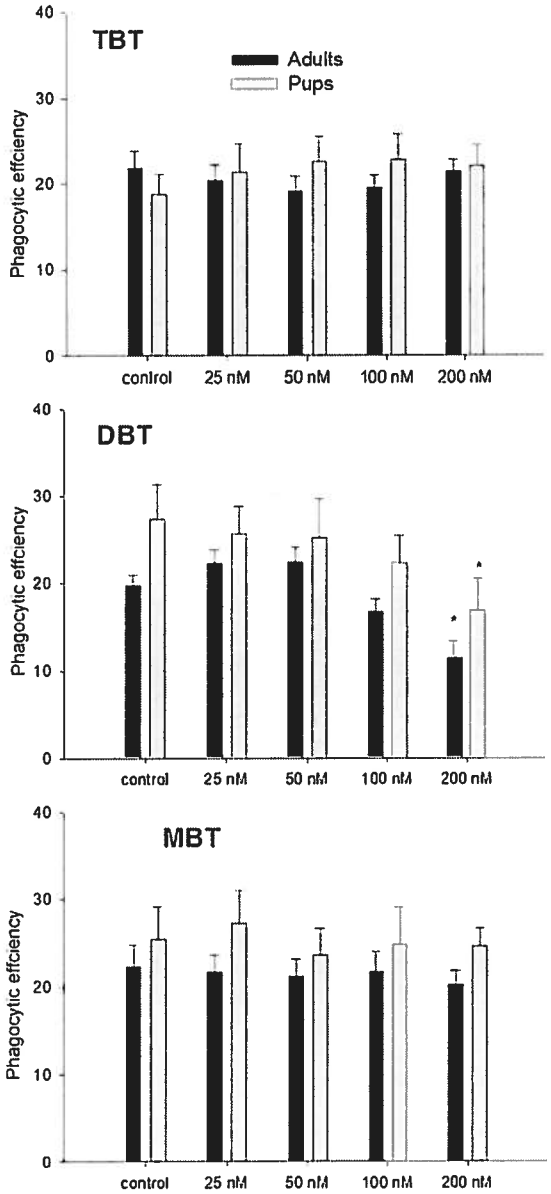

Fig. 3. Effects of butyltins, TBT, DBT and MBT on the efficiency of phagocytosis. Values are calculated as the percentage of macrophages having engulfed three beads or more. Results are given as mean ± standard error. Asterisk shows a statistical difference from control cells ($P < 0.05$).

4. Discussion

In this study, we evaluated the contamination and the ecotoxicological effects induced by butyltins on the immune system using chemical analysis coupled to traditional immunology methods. Cell viability was shown to be unaffected by TBT, DBT or MBT concentrations used in this experiment. This indicates that the adverse effects observed were not the result of decreased cell viability.

4.1. Concentration levels of TBT and its metabolites

We detected low butyltin concentrations in blood and blubber of harbour seal pups from Bic National Park. This observation is consistent with results from a previous study showing the presence of low, but ubiquitous, concentrations of butyltins in fish collected in the St. Lawrence Estuary (Michaud and Pelletier, 2006). Therefore, a low contamination in offspring could either indicate a low

Table 3
Immunosuppression dosage at 50% proliferation response of butyltins in adult (male and female) and pup harbour seals.

Compounds	Adults		Pups	
Monobutyltin	–		–	
Dibutyltin	Mean 144 nM		160 nM	
Details	Males 151 nM (2)	Females 141 nM (5)	Males 169 nM (5)	Females 72 nM (1)
Tributyltin	Mean 310 nM		–	
Details	Males 345 nM (3)	Females 286 nM (5)		

Number of samples is given in brackets. (–) Indicates that the proliferation response did not decrease below 50% for the range of tested concentrations.

Table 4
Butyltin concentrations (ng Sn/g ww) in the blubber of pinnipeds from various areas.

Species	Location	MBT	DBT	TBT	ΣBTs	*n*	Age (years)	Sex	Sources
Harbour seal *Phoca vitulina*	St. Lawrence Estuary, Canada	1	<0.04	5.5	6.5	6	1–10 days	M = 5, F = 1	This study
Harbour seal *Phoca vitulina*	Norway	3.3	<3	<3	3.3	10	No data	M = 9, F = 1	Berge et al. (2004)
Larga seal *Phoca largha*	Hokkaido, Japan	<14	<1	4.4	4.4	1	No data	M	Iwata et al. (1994)
Ringed seal *Phoca hispida*	Norway	<1	<1	<1	<1	2	No data	No data	Berge et al. (2004)
Steller Sea Lion *Eumetopias jubatus*	Hokkaido, Japan	7.1	5.2	5.6	18	1	4.5	M	Kim et al. (1996)

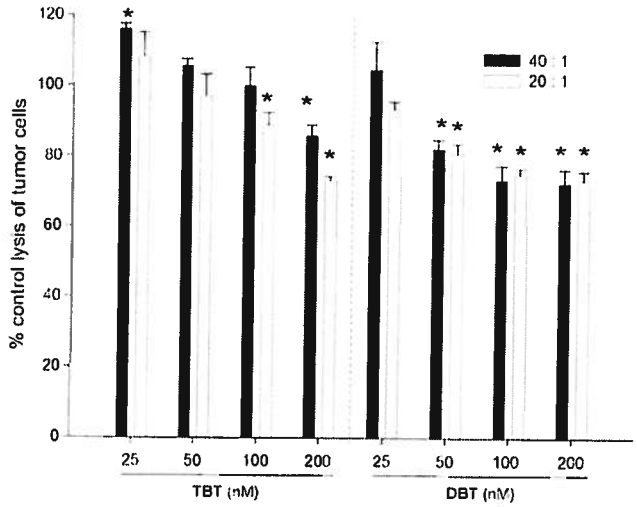

Fig. 4. Effects of BTs exposure on the ability of NK cells to lyse tumor cells. $^{*}P \leq 0.05$. The level control (100%) represents the lytic function the control cells (without butyltin compounds). Combined results from three separate experiments (mean ± standard error) using three adult female seals.

contamination in seal mothers or a low maternal transfer of BTs. TBT concentration levels found in our study were similar to those of pinnipeds from other part of the world (Table 4). The DBT and MBT concentrations observed in blubber of harbour seals from St. Lawrence Estuary were generally lower than those observed in other pinnipeds. The concentration sequence for BTs found in our study can be arranged as follows: TBT > MBT > DBT. One part of TBT in the pups might have been metabolized into its breakdown products. This could explain some individual differences in BTs concentrations. The complete metabolism of TBT is expected to result in higher concentrations of MBT and DBT. Elsewhere, regarding BTs composition, a higher proportion of metabolites (DBT and MBT) rather than TBT is found in liver of adult pinnipeds (Kim et al., 1996). However, in our study, DBT was not detected. Another study observed exactly the same pattern in the liver tissue of two grey seals (Ciesielski et al., 2004). The two seal specimens were pups of the age of 1.5 and 3 months, respectively and DBT was not detected. So, it seems that the absence of DBT could be linked to age. However, the absence of DBT in pups remains to be explained. This finding may be an indication of a limited transfer of BTs by harbour seal mothers to their offsprings *via* placenta and breast milk, and suggests that DBT has lower placental and milk transfer potential than TBT and MBT in this species. This differs from killer whale (*Orcinus orca*) calves, where DBT was the dominant component in the liver, implying that BTs are transferred through gestation/lactation

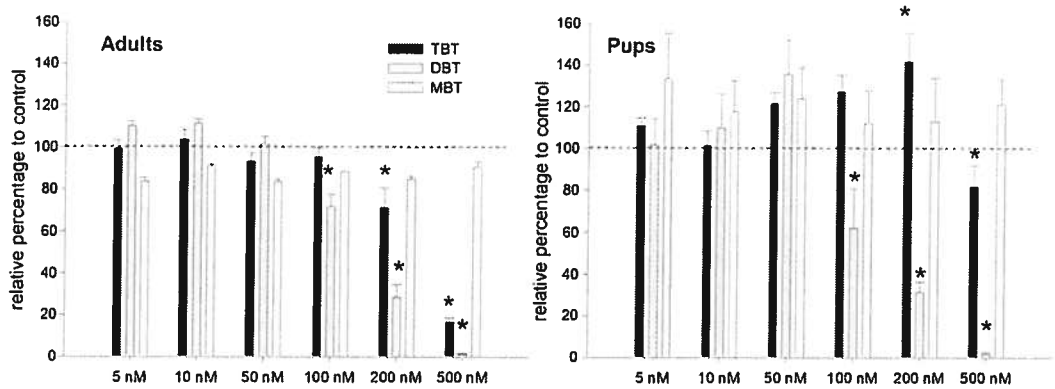

Fig. 5. Proliferation response of peripheral blood mononuclear cells (PBMCs) in adult and pup seals following the treatment with BTs. $^{*}P \leq 0.05$. The level control (100%) represents results from peripheral blood mononuclear cells cultures without butyltin compounds.

from mother to fetus/pups in toothed whales (Kajiwara et al., 2006).

To our knowledge, no previous study has evaluated the BTs levels in blood of pinnipeds. In our study, concentrations of BTs in blubber and blood of harbour seal pups were different and ranged from 1.2 to 13.4 and from 0.1 to 0.4 ng Sn/g ww, respectively. Moreover, the proportions of TBT, DBT and MBT varied between blood and blubber tissues. This observation corroborates a report by Iwata et al. (1997) who found that the proportions of TBT, DBT and MBT in finless porpoise (*Neophocaena phocaenoides*) samples were different from one organ/tissue to another. In adult finless porpoises, DBT comprised a higher proportion in the liver with 1800 ng butyltin ion/g ww (740 ng Sn/g ww) representing 55–71% BTs and in the blood with 640 ng butyltin ion/g ww (260 ng Sn/g ww) representing 78% BTs in comparison to other tissues and organs (notably in blubber where residue levels of DBT were lower than MBT and TBT concentrations). Considering the presence of a higher proportion of DBT residues compared to TBT in blood from adult marine mammals, it was justified to examine the toxicity of DBT on the immune system including innate immunity (phagocytotic activity and NK cell activity) and humoral immunity (lymphocyte proliferation).

4.2. Effects of age and gender on immune functions

Many of the age-related phenomena that affect the immune system had been recognized for some time. Cellular and humoral immune responses in neonates differed both qualitatively and quantitatively from those of adults. We observed that phagocytic activity was significantly more important in pups than in adults. This observation differed from McKay and Lu (1991) who found that macrophage function appeared to be impaired in the neonate, possibly as a result of inhibitory factors present in the serum. However, Ross et al. (1994) have speculated that the relative immunocompetence of the harbour seal at birth reflects an adaptation to its relatively short nursing period and limited maternal care. Moreover, Menge et al. (1998) showed in all species where maternal transfer of immunoglobulins occurs primarily or exclusively through the colostrum, that ingestion of colostrum greatly influences the performance of phagocytes in newborns. Sugisawa et al. (2001) showed that phagocytosis-promoting factor(s) are present in bovine colostrum as demonstrated in the case of human colostrum (Straussberg et al., 1995).

Our study also demonstrated that *in vitro* exposure to BTs decreased lymphocyte proliferation differently in males and in females. In adults, females were more sensitive to BTs, notably to TBT, than males. Pillet et al. (2000) demonstrated that exposure of PBLs to $ZnCl_2$ resulted in a gender-specific response and that PBLs from mature female seals were more sensitive to $ZnCl_2$ than those of either male or immature seals. These authors suggested that immune cells from female seals may be differently preconditioned by *in vivo* exposure to female sex hormones.

4.3. Immunological impact of butyltins

In our experiment, TBT and MBT had no effect on phagocytosis in adults and pups. The phagocytic activity of adults PBLs was impaired at both 100 and 200 nM concentrations of DBT and the phagocytic efficiency was affected at 200 nM DBT. However, in pups, the DBT inhibited only the phagocytic efficiency at 200 nM. No other studies examined the effects of DBT and TBT on phagocytic activity using a mammalian model, but using an invertebrate model, these results are consistent with published reports on higher toxicity of DBT versus TBT at 1 μM (Bouchard et al., 1999; Cima et al., 1995) but inconsistent with one other report (Cooper et al., 1995), which showed that TBT at 1.5 μM affected phagocytic activity more markedly than did DBT.

TBT and DBT exposure decreased NK cell activity. The observation of inhibited NK cell activity agrees with previous studies reporting reduced NK cell activity in both *in vivo* and *in vitro* exposure schemes to TBTCl and TBT oxide (TBTO) (Ghoneum et al., 1990; Smialowicz et al., 1990; Van Loveren et al., 1990; Whalen et al., 1999, 2002). However, in those studies, *in vitro* toxicity of TBT on NK cells was stronger than the effects we observed at the same concentrations. The suppression of cytotoxic function by TBT could be attenuated by presence of T lymphocytes (Wilson et al., 2004). The consistently enhanced NK cell activity that we observed with the lowest concentrations of TBT was unexpected and is in contrast to previous studies. Nevertheless, a recent *in vivo* study observed enhancing effects of TBTCl on NK cell activity only in the low dose group (0.025 mg/kg bw/day) in old female rats (Tryphonas et al., 2004). The significantly inhibited NK cell activity observed at 50, 100 and 200 nM of DBT is in contrast to the findings of a previous study, in which the authors observed that after a 24-h exposure to 0.5 μM and higher of DBT, the human NK cytotoxic function was reduced by about 30% or more (Whalen et al., 1999).

Among adult seals, the inhibition levels observed with 100 nM of DBT and 200 nM of TBT were similar to those reported by Nakata et al. (2002) on the immunotoxic effects of butyltins on marine mammal (pinnipeds and cetaceans) lymphocytes. These authors observed that con A-stimulated mitogenesis were significantly suppressed when cells from pinnipeds, a California sea lion (*Zalophus californianus*) and a larga seal (*Phoca largha*), were exposed to 300 nM of TBT and 330 nM of DBT. In fish, a high concentration of TBT or DBT (500 ppb) induced a suppression of the humoral immune response of more than 95% in both head kidney and spleen leukocytes (O'Halloran et al., 1998).

Our results demonstrate a different proliferative response in pup and in adult cells after exposure to BTs. Harbour seal pups (1–10 days old) have been shown to be immunocompetent, as they demonstrated an *in vivo* antibody response to immunization with an inactivated rabies vaccine and by strong *in vitro* lymphocyte proliferation responses to mitogens (Con A, PHA and pokeweed mitogen), suggesting an evolutionary adaptation to its short nursing period (~24 days) and limited maternal care (Ross et al., 1994). Ross et al. (1993, 1994) have reported that lymphocyte response to Con A in harbour seal pups are low at birth and higher at the end of lactation and pup lymphocytes respond stronger to Con A than the lymphocytes of their mothers. In our study, the use of harbour seals of approximately 1–16 days of age, which presumably already have developed a competent immune system, could have implicated age as a cofactor affecting lymphocyte proliferation. Levin et al. (2005) demonstrated an immunostimulation linked to polychlorinated biphenyls in free-ranging harbour seal pups. Moreover, Lalancette et al. (2003) reported that lymphoblastic transformation in grey seals was more affected by MeHgCl contamination after the complete development of immune system than before. Our study suggests that free-ranging harbour seal pups may experience a period of BT-associated immunostimulation at the relatively low-dose range used. A decrease in immune function can have devastating effects on the survival of an organism, particularly during early critical phases of development. Whereas chemical-induced immune suppression may result in increased susceptibility, incidence and severity of infectious diseases, conversely, immune enhancement may result in the loss of regulation within the immune system and can lead to adverse outcomes including autoimmune disease, anergy, and cancer (Descotes et al., 2000).

The higher immunotoxicity of DBT compared to TBT might be linked to at least three factors acting simultaneously: the alter-

ation of Ca^{2+} efflux by DBT, the particular membrane affinity of TBT and the presence of the operon tbtABM. The immunotoxicity of butyltins could be explained by a rapid increase in the intracellular Ca^{2+} concentration in immune cells caused by TBT (Chow et al., 1992) and DBT (Gennari et al., 2000) resulting in a modification of cellular structure and functions (Cima et al., 1998). The second possible explanation could be the partial retention of TBT at the surface or within the plasma membrane of leukocytes, as was demonstrated by Porvaznik et al. (1986) in human erythrocytes at or above 10 μM TBT concentrations. DBT failed to produce tin-containing aggregates in cell membranes at 5000 μM (Gray et al., 1987). The partial retention of TBT in membranes coupled with better competition to Ca^{2+} sites by DBT may provide an explanation for the different potency of the two-organotin compounds studied. A third explanation could be based on the presence of an operon, called *tbtABM*. Jude et al. (2004) demonstrated that TbtABM is responsible for the TBT resistance in *Pseudomonas stutzeri* and *Escherichia coli* due to an active efflux of this toxic compound out of the cells. Despite the absence of direct evidence in immune cells, we also suggest that this efflux pump (TbtABM) removes TBT out of these cells, which could explain the lower toxicity of this compound.

Concentrations of BTs measured in blood of wild harbour seal pups from Bic National Park were to low to induce toxicity to their immune system during first days of life. Further investigation is needed particularly in the analysis of BTs levels in blood from adult pinnipeds. However, based on our *in vitro* results, we hypothesize that in adults, blood concentrations of BTs, and DBT in particular, might induce immunotoxic effects on phagocytosis, cytotoxic activity of NK cells or lymphocyte proliferation.

We have demonstrated that exposure to DBT significantly reduce immune functions of harbour seal PBLs *in vitro*, and that TBT had less effect on the same cells. This finding has implications for the health of this species in response not only to BTs but other persistent contaminants that are still accumulating in the environment and that pose a serious threat to the immune system of free-ranging marine mammals. It is possible that different species sharing the same environment show different sensitivities to BT compounds. However, the mechanisms by which such differences arise are still unknown.

Acknowledgements

This work was supported by the Canada Research Chair in Environmental Immunotoxicology (M.F.). This research project was funded in part by the Canadian Research Chair in Marine Ecotoxicology (E.P.), the Natural Sciences and Engineering Research Council of Canada (E.P.) and the Department of Fisheries and Oceans (M.L.). Many thanks to the field crew for their hard work and enthusiasm, most-notably: Steve Trottier, Yves Dubé and Jean-Francois Gosselin. The expert technical assistance of Marlene Fortier was greatly appreciated. The authors wish to thank the staffs of the aquarium at Shippagan and the zoo at St.-Félicien for providing blood samples from the marine mammals. The manuscript benefited from valuable discussion and comments provided by Dr. S. Pillet.

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5.2. Les effets *in vitro* de congénères individuels de polybromés diphényles éthers (PBDE) sur les cellules immunitaires de phoque commun

Article 7: *Héloïse Frouin, Michel Lebeuf, Mike Hammill, Stéphane Masson, Michel Fournier. Effects of individual polybrominated diphenyl ether (PBDE) congeners on harbour seal immune cells in vitro. Marine Pollution Bulletin. (Article accepté le 4 septembre 2009).*

Mon rôle a consisté à faire l'échantillonnage, les analyses, interpréter les données et écrire cet article à titre d'auteur principal.

RÉSUMÉ

Les effets des polybromés diphényles éthers (PBDE) sur le système immunitaire des mammifères marins sont peu compris. Une fonction immunitaire innée importante des granulocytes est la flambée oxydative qui génère des espèces oxygénées réactives (EOR) utilisées pour tuer les microorganismes ingérés. La présente étude analyse *in vitro* les effets du BDE-47, -99 et -153, sur la formation des EOR, sur le niveau intracellulaire de thiols, sur l'activité et l'efficacité phagocytaire et sur l'apoptose chez les granulocytes de phoque commun. Les composés ont été testés à quatre concentrations différentes allant de 1.5 à 12 µM. Les résultats montrent que les niveaux de EOR, les niveaux de thiols et la phagocytose sont affectés quand les cellules de phoque commun sont exposées à la plus forte concentration (12 µM) des congénères de PBDE. L'apoptose n'est pas affectée par les PBDE. Les effets observés étaient similaires entre les phoques commun adultes et nouveau-nés et la lignée cellulaire 11B7501 issue d'un phoque commun.

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Marine Pollution Bulletin xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Effects of individual polybrominated diphenyl ether (PBDE) congeners on harbour seal immune cells *in vitro*

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ARTICLE INFO

Keywords:
PBDEs
Granulocytes
Oxidative stress
Phagocytosis
Cell line
Harbour seals

ABSTRACT

Effects of polybrominated diphenyl ethers (PBDEs) on the immune system of marine mammals are poorly understood. One important innate immune function of granulocytes is the respiratory burst which generates reactive oxygen species (ROS) used to kill engulfed microorganisms. The present study investigates *in vitro* the effects of BDE-47, -99 and -153, on the formation of ROS, on intracellular level of thiols, on activity and efficiency of phagocytosis and on apoptosis in granulocytes of harbour seals. Compounds were tested at four different concentrations ranging from 1.5 to 12 μM. Results showed that ROS levels, thiol levels and phagocytosis were all affected when harbour seal cells were exposed to the highest concentration (12 μM) of PBDE congeners. Apoptosis was not affected by PBDEs. The observed effects were similar in adults, pups and in the 11B7501 cell line of harbour seals.

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1. Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants (BFRs) made up of 209 possible congeners containing between 1 and 10 bromine atoms (Alaee et al., 2003). PBDEs are not covalently bound to the polymers during their formation, and can thus leak into the environment (Alaee et al., 2003; Hale et al., 2001; Rahman et al., 2001). There are three types of commercial PBDE products including the penta-BDE mixture (DE-71 and Bromkal 70-5DE), the octa-BDE mixture (DE-79 and Bromkal 79-8DE) and the deca-BDE product (Saytex 102E and Bromkal 82-ODE). However, due to growing environmental and human health concerns, two of the three commercial formulations (Penta and Octa) were removed from the European (1998) and North American (2004) marketplace (Ross et al., 2009). Deca-BDE formulation was removed in Europe in 2008 but is poised for a ban in the national markets in Canada and the USA (some US states have regulated Deca-BDE) (Ross et al., 2009).

Recent studies have shown that PBDEs are ubiquitous in freshwater and marine environments (De Wit, 2002; Wolkers et al., 2004a). Among the PBDE congeners that have been detected in biota, the most common are 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99), 2,2′,4,4′,6-pentabromodiphenyl ether (BDE-100) all found in penta-BDE mixture and 2,2′,4,4′,5,5′-pentabromodiphenyl ether (BDE-153) also found in octa-BDE mixture (La Guardia et al., 2006).

Total PBDE concentrations are increasing rapidly in abiotic and biotic matrices in Canada, and have surpassed the structurally-related PCBs as the number one contaminant in sewage, sediments and water in near-urban parts of Canada. In extrapolating recent trends, PBDEs are projected to surpass PCBs as the top contaminant in many fish and marine mammal populations within 10 years (Ikonomou et al., 2002; Ross, 2006). In eastern Canada, the St. Lawrence Estuary, which is located downstream of the Great Lakes–St. Lawrence River, receives the waters from one of the most industrialised regions of the world. PBDEs were detected in the blubber of adult (Law et al., 2003) and in the blubber and blood of pup harbour seals from the St. Lawrence Estuary (Frouin et al., 2009).

Marine mammals due to their life cycle are particularly sensitive to the effects of contaminants accumulating mainly through their diet (Borga et al., 2004). For example, seals reduce their food intake during lactation, molting and breeding, resulting in the mobilization of body fat to meet energy demands. It has been shown that with lipid mobilization from body fat, contaminants that have accumulated in main fat depots (blubber) during the preceding months are also mobilized, resulting in increased blood contaminants levels (Lydersen et al., 2002). In marine mammals, transplacental and lactational transfer of organic contaminants may affect offspring at a critical stage of their development (Duinker and Hillebrand, 1979; Wolkers et al., 2004b).

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doi:10.1016/j.marpolbul.2009.09.006

Please cite this article in press as: Frouin, H., et al. Effects of individual polybrominated diphenyl ether (PBDE) congeners on harbour seal immune cells *in vitro*. Mar. Pollut. Bull. (2009), doi:10.1016/j.marpolbul.2009.09.006

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Immunotoxicity of PBDEs was previously reported (Fernlof et al., 1997; Fowles et al., 1994; Martin et al., 2007; Thuvander and Darnerud, 1999). While the results of these studies may shed some light on the ability of PBDEs to present some aspects of immunotoxicity, their effects on the immune function of marine mammals at this point remain largely unknown. However, Beineke et al. (2005) demonstrated that thymic atrophy and splenic depletion in harbour porpoises (*Phocoena phocoena*) were significantly correlated to increased PBDE levels.

The exposure to persistent contaminants such as PBDEs induces oxidative stress in humans (Reistad and Mariussen, 2005) and results in changes in antioxidant stores and enzymatic activity as demonstrated in birds (Fernie et al., 2005). Cellular defence mechanisms such as glutathione metabolism, thiols and lipid peroxidation serve as sensitive measures of oxidative stress when animals are exposed to structurally-related polychlorinated biphenyls (Palace et al., 1997).

The aim of this study was to investigate the toxicity of three PBDEs (BDE-47, -99 and -153) in harbour seal (*Phoca vitulina*) cells and in 11B7501 cells, supplying data to increase our understanding of the potential role of environmental pollutants in the seals. The 11B7501 B lymphoma cell line was purchased from the American Type Culture Collection (CRL-1940) and was used as a model in this study. It was developed from the peripheral blood mononuclear cells derived from a male harbour seal (*P. vitulina*) with malignant leukemia lymphoma. Results obtained with cell line will be compared to results observed with harbour seal cells *in vitro*. This is the first study to report the toxic effects of PBDEs in harbour seals.

2. Materials and methods

2.1. Animal capture, handling and sample collection

Six pup harbour seals *P. vitulina* L. were sampled in June 2008 at Bic Island (near Bic National Park), along the south shore of the St. Lawrence Estuary in Quebec, Canada (Table 1). Seals were captured in the water using an inflatable boat and a dip net and were subsequently transferred to a larger boat where all handling took place. Prior to analysis, seals were weighed (to ±0.5 kg), tagged, and their sex noted (Dubé et al., 2003). All handling of the pup was carried out as quickly as possible (within 20 min) to avoid abandonment of the pup by the mother (Boulva and McLaren, 1979). Pups were captured throughout the lactation period. Blood samples, between 10 and 20 mL, were taken from the extradural intravertebral vein into Vacutainer tubes (Becton–Dickinson, New Jersey, USA) and were immediately stored at 4 °C until analysis (6 h after sampling). The volume of blood collected was no more than 10% of the total blood volume (McGuill and Rowan, 1989). All research was approved by the Animal Care Committees Fisheries and Oceans, Canada.

Fresh blood samples were also collected from eight harbour seal adults housed in aquaria (Zoo de Saint-Félicien, Canada and Aquarium du Québec, Canada) (Table 1). These samples were taken from a superficial plantar flipper veins and approximately 10–20 mL of whole blood was collected from each animal. Blood was kept at 4 °C until analysis, 6–8 h after sampling.

2.2. Isolation of peripheral blood leukocytes (PBL)

PBL were isolated using the protocol described by Frouin et al. (2008). The cells were resuspended in RPMI-1640 (Sigma) supplemented with 10% heat inactivated foetal bovine serum (FBS), 1% Pen-Strept and 10 mM Hepes and kept overnight at 4 °C. The ratio of live/dead cells was assessed using trypan blue dye exclusion and visual examination under a microscope with a hemacytometer. Cell viability was always greater than 90%. The suspension was adjusted to 10^6 live cells/mL before use. This method of isolation isolates all leukocytes subpopulations (i.e., lymphocytes, granulocytes and monocytes) (Frouin et al., 2008).

2.3. Cultured cell line

Scattergram of the flow cytometric profiles of the cell suspension (11B7501 cell line) indicates that two subpopulations (i.e., B/macrophage cells and lymphocytes) were present in this cell suspension. This phenomenon was previously described in several malignant B-cell lineage and B/macrophage cells were described as highly phagocytic (Borrello and Phipps, 1996). The cell line was grown in continuous culture in complete RPMI-1640 medium supplemented with 10% FBS, 1% Pen-Strept and 10 mM Hepes.

2.4. Chemical preparation

2,2′,4,4′-Tetrabromodiphenyl ether (BDE-47, purity 99.5%), 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99, purity 99.6%) and 2,2′,4,4′,5,5′-hexabromodiphenyl ether (BDE-153, purity 99.8%) were obtained from the Cambridge Isotope Laboratories Inc. (Andover, MA, USA). The purity of these compounds was confirmed by GC/MS. Stock solutions were prepared by dissolving each compounds in DMSO. The final concentration of DMSO in the samples was always less than 1%. This DMSO concentration showed no effects on function cell. Borosilicate glassware was used to avoid the adsorption onto the polypropylene round bottom tubes in the sample container during exposure treatment. Working dilutions of PBDE treatments were prepared in complete RPMI for the nominal concentrations 1.5, 3, 6 and 12 μM (or DMSO control).

2.5. Viability

In each tube, 50 μL of PBDE treatment solutions or vehicle control was added with 500 μL of the cell suspension (10^6 cells/mL). Viability of cells after exposure was evaluated by flow cytometry using propidium iodide (PI) (100 μg/mL) as described by Brousseau et al. (1999). PI enters in all cells but is actively excreted only by living cells. Samples were analyzed with a FACScalibur flow cytometer (Becton–Dickinson, San José, USA) and the fluorescence of 5000 events was read in FL3. Data are expressed as a percent of viable cells.

Table 1
Description of investigated seals.

Species	Date of collection (2008)	Location (Canada)	*n*	Sex[a]	Age	Tissues analyzed
Harbour seal (*Phoca vitulina*)	June	Zoo (Saint-Félicien)	4	F:4	Adult	Blood[b]
Harbour seal (*Phoca vitulina*)	June	Bic National Park	6	M:2, F:4	Pup	Blood[b]
Harbour seal (*Phoca vitulina*)	October	Aquarium du Québec	4	F:4	Adult	Blood[b]

[a] M (male); F (female).
[b] Fresh samples.

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2.6. Phagocytosis

Phagocytosis was shown to be a good biomarker of immunotoxicity in wildlife species (Fournier et al., 2000). Fifty microliters of a PBDE treatment solutions or vehicle control was added to the appropriate tube. Five hundred microliters of the suspension of 10^6 cells/mL were added to the PBDE-loaded tubes. The cells were exposed to the test compounds in a humidified incubator at 37 °C with 5% CO_2 for 24 h. The measurement was performed as described elsewhere (Brousseau et al., 1999). Cells were incubated with fluorescent latex beads during 3 h. The samples were then analyzed with a FACScalibur flow cytometer (Becton–Dickinson, San José, USA) equipped with a 488-nm argon laser. Granulocyte population was determined by gating on a forward scatter (FSC) and side scatter (SSC) dot plot as described previously. For each sample, the fluorescence of 10,000 events was recorded. Results were analyzed with the Cell Quest Pro software (Becton–Dickinson) to determine the percentage of PBLs that engulfed one bead and more (phagocytic activity) or three beads and more (phagocytic efficiency). The absence of fluorescent response observed in lymphocytes corresponds to their inability to perform phagocytosis and represents an appropriate negative control. The results were expressed as the percentage of phagocytosis.

2.7. Free radical production

The production of ROS, such as hydrogen peroxide and superoxide anions was quantified by measuring the oxidation of 2,7 dichlorofluorescein diacetate (DCF-DA) at baseline and after cell stimulation with phorbol myristate acetate (PMA) [protocol modified from Brousseau et al. (1999)]. For each sample, two 500 μL volumes were prepared at a cell concentration of 10^6 mL^{-1} and 50 μL of PBDE treatment solutions or vehicle control was added. After an incubation of 24 h, cells were collected and resuspended in 500 μL sterile PBS-G (PBS with 1 g/L glucose) with 10 μL 5 mM DCF-DA, which was permeable across cell membranes and incubated at 37 °C for 45 min in the dark. Inside the cell, the acetate moiety was cleaved by cellular esterase and DCFH readily reacted with ROS to form the fluorescent DCF. After labelling, leukocytes were incubated for 15 min at 37 °C in the presence or absence of 50 μL 1 μM of PMA. Total leukocytes were subjected to flow cytometry analysis for measurement of intracellular ROS production by granulocytes. Fluorescence emission was collected at 520 nm and a total of 10,000 events were acquired for each sample. The results are expressed as the ratio in mean fluorescence between the control (DCF-DA only) and the stimulated cells (DCF-DA + PMA) demonstrating the intensity of the respiratory burst.

2.8. Determination of the cellular thiol status

The intracellular thiol concentration was measured by CMFDA staining in flow cytometry according a method modified from Brousseau et al. (1999). After exposure during 24 h (as described above), cells were collected and resuspended in 500 μL sterile PBS-G and cell samples were stained with CMFDA at a final concentration of 50 μM for 45 min at 37 °C in the dark. The cells were analyzed immediately after by flow cytometry. Green fluorescence from CMFDA was measured in the FL1 channel through a 525 nm band-pass filter. The level of intracellular thiols was indicated by mean fluorescence intensity of stained probes.

2.9. Assessment of apoptosis

Apoptotic cells were counted by flow cytometry. For staining with the impermeant nuclear dye YO-PRO-1/propidium iodide (Vybrant Apoptosis Assay Kit #4, Molecular Probes, USA), cells after exposure to PBDEs (as described above) were collected and resuspended in 500 μL sterile cold PBS with 200 μL of stain (prepared according to manufacturer's instructions). Staining was carried out at 4 °C for 30 min in the dark. At least 4000 cells were counted for each sample over a constant period of time. Dexamethasone was used as an inducer of apoptosis at 1 μM.

2.10. Statistical analyses

Correlation of tested parameters and BDE-47, -99 and -153 concentrations were analyzed by linear regression. The calculations were performed using STATISTICA for windows (version 7.0, StatSoft Inc., 1995).

3. Results

3.1. Viability

There were no statistically significant treatment-related effects on the viability of immune cells (data not shown). The percentage of viability in control samples were 95.53% ± 0.04 (mean ± SEM).

3.2. The effects of different PBDEs on phagocytosis of seal granulocytes

The BDE-47, -99 and -153 induced a concentration-dependent decrease of phagocytosis in seal granulocytes (Fig. 1). In adults, pups, and cell line, a similar inverse correlation between phagocytic activity and BDE-47 ($r^2 = 0.84$, $p = 0.03$; $r^2 = 0.80$, $p = 0.04$; $r^2 = 0.98$, $p = 0.001$, respectively), BDE-99 ($r^2 = 0.84$, $p = 0.03$; $r^2 = 0.88$, $p = 0.02$; $r^2 = 0.99$, $p = 0.0001$, respectively) and BDE-153 ($r^2 = 0.92$, $p = 0.009$; $r^2 = 0.90$, $p = 0.01$; $r^2 = 0.98$, $p = 0.001$, respectively) concentrations was found while a similar inverse correlation between phagocytic efficiency and BDE-47, -99 and -153 concentrations was found only in pups ($r^2 = 0.83$, $p = 0.03$; $r^2 = 0.95$, $p = 0.006$; $r^2 = 0.86$, $p = 0.02$, respectively) and cell line ($r^2 = 0.94$, $p = 0.006$; $r^2 = 0.99$, $p = 0.0004$; $r^2 = 0.94$, $p = 0.006$, respectively).

3.3. Increased intracellular H_2O_2 accumulation in PBDEs-exposed seal neutrophil granulocytes

PBDEs induced a concentration-dependent increase in ROS formation in seal neutrophil granulocytes (Fig. 2). A similar correlation was found between ROS formation and BDE-47, -99 and -153 concentrations in adults ($r^2 = 0.91$, $p = 0.01$; $r^2 = 0.97$, $p = 0.002$; $r^2 = 0.89$, $p = 0.02$, respectively) and pups ($r^2 = 0.97$, $p = 0.002$; $r^2 = 0.97$, $p = 0.003$; $r^2 = 0.95$, $p = 0.005$, respectively). No ROS formation was detected in harbour seal cell line (data not shown).

3.4. Thiol depletion induced by PBDEs

Intracellular thiol levels decreased after exposure to BDE-47, -99 and -153 in adults, pups and cell line (Fig. 3). A similar inverse correlation between thiol levels and BDE-47, -99 and -153 concentrations was found in adults ($r^2 = 0.97$, $p = 0.003$; $r^2 = 0.95$, $p = 0.005$; $r^2 = 0.99$, $p = 0.00005$, respectively), pups ($r^2 = 0.98$, $p = 0.001$; $r^2 = 0.96$, $p = 0.003$; $r^2 = 0.95$, $p = 0.004$, respectively) and cell line ($r^2 = 0.99$, $p = 0.00005$; $r^2 = 0.88$, $p = 0.02$; $r^2 = 0.86$, $p = 0.02$, respectively).

3.5. Effects of PBDEs on apoptosis

Results show that treatment of harbour seal cells and harbour seal cell line with all nominal concentrations (1.5, 3, 6 and

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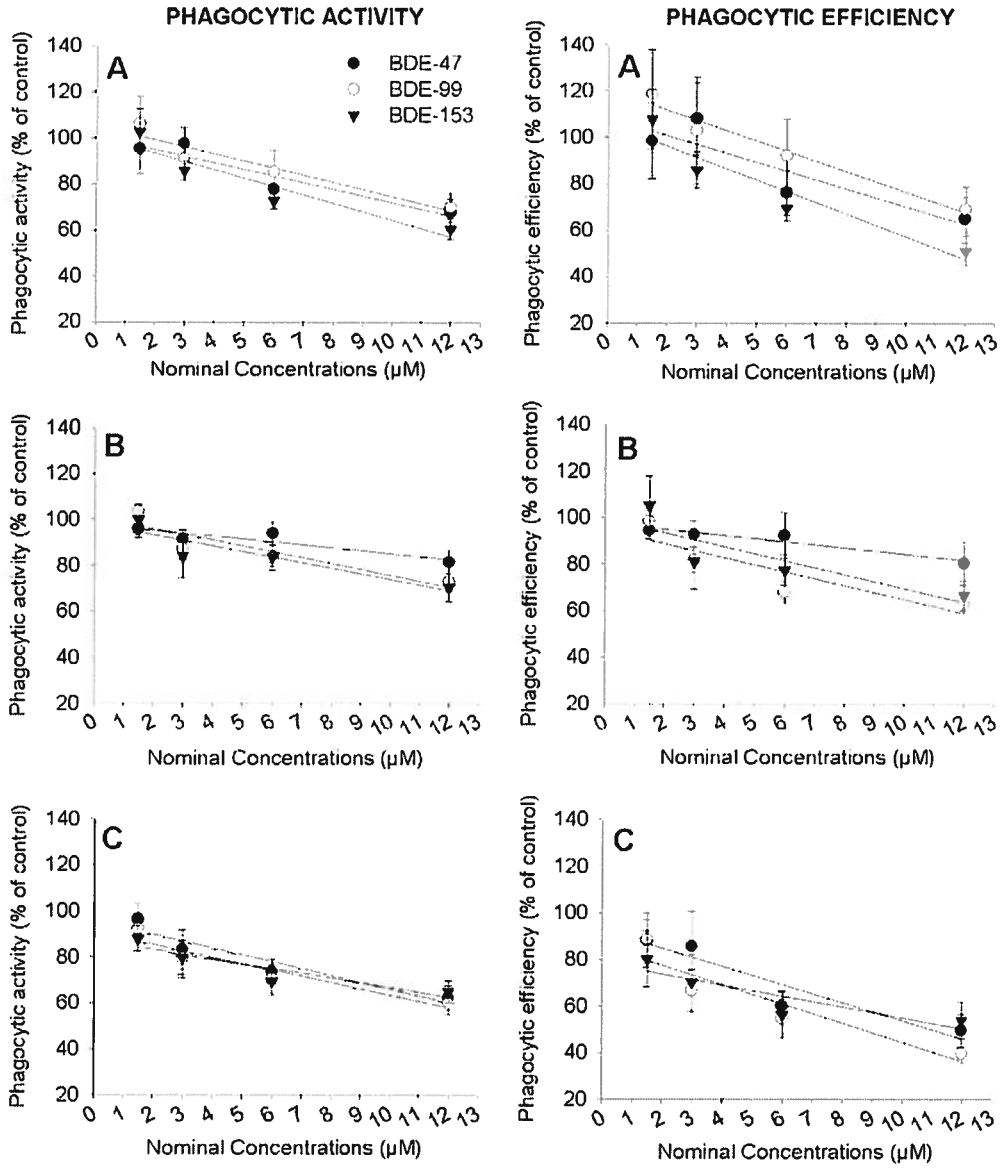

Fig. 1. Significant negative correlations existed between PBDE congeners (BDE-47, BDE-99 and BDE-153) and the activity and efficiency of phagocytosis in adults (A), pups (B) and 11B7501 cell line (C). The percentage of phagocytosis after 24 h of exposure was determined by flow cytometry after incubation with latex beads. All values are presented as the percentage of control (vehicle control). Values are mean ± SEM. Phagocytic activity is defined as the percentage of cells that phagocytised one bead and more, whereas phagocytic efficiency is defined as the percentage of cells that phagocytised three beads and more.

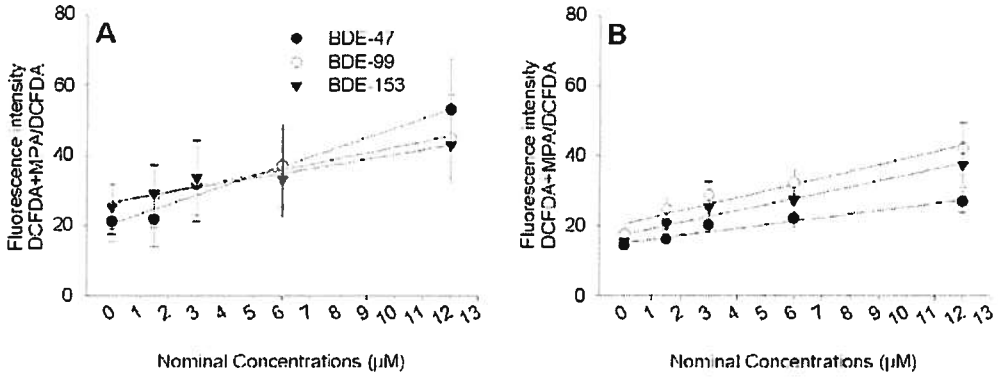

Fig. 2. Significant negative correlations existed between PBDE congeners (BDE-47, BDE-99 and BDE-153) and the ROS level in harbour seal cells (A: adults and B: pups). The production of ROS after 24 h of exposure was determined by using DCFH-DA, which is permeable across cell membranes and readily reacts with ROS to form the fluorescent DCF. The fluorescent DCF intensity reflects ROS level of cells at baseline and after cell stimulation with PMA. Values are presented as the mean ± SEM.

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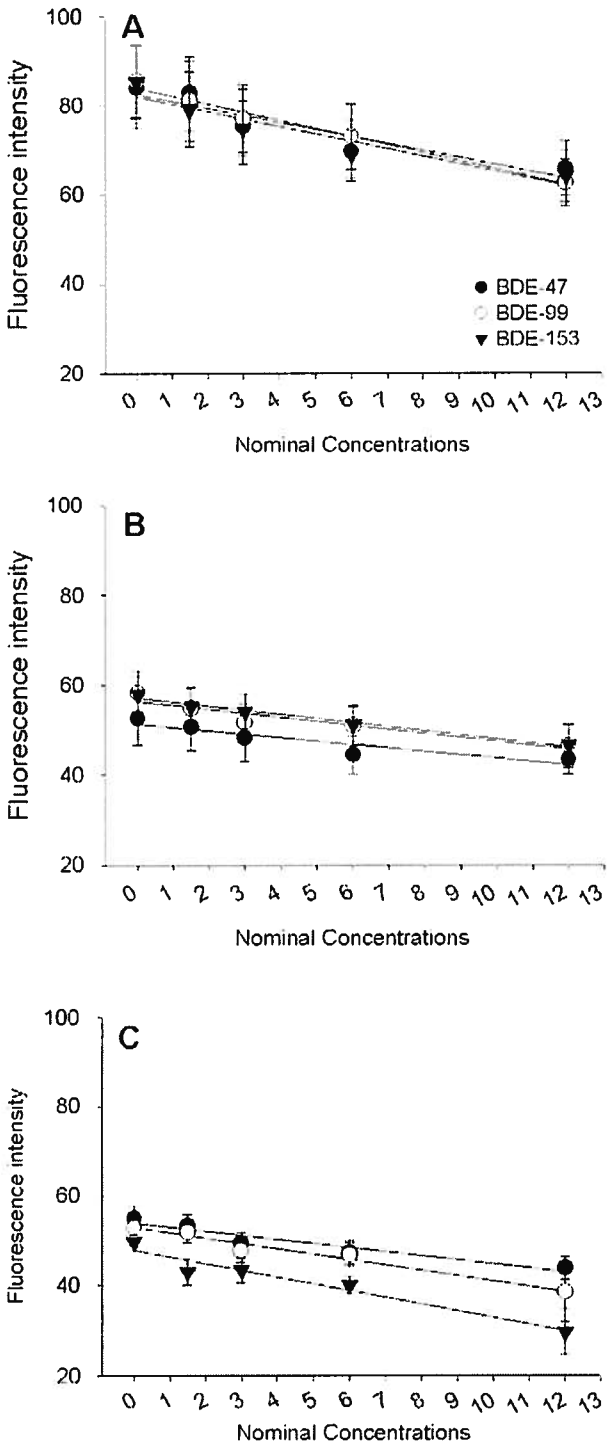

Fig. 3. Significant negative correlations existed between PBDE congeners (BDE-47, BDE-99 and BDE-153) and the thiol concentration in adults (A), pups (B) and cell line (C). Thiol intracellular level after 24 h of exposure was determined using CMFDA. Values are presented as the mean ± SEM.

12 μM) did not significantly change the percentage of apoptotic cells (Fig. 4, only with 12 μM).

4. Discussion

Results presented in this study clearly demonstrate that brominated flame retardants are able to induce thiol depletion and phagocytosis inhibition in harbour seal immune cells *in vitro* and harbour seal cell line and ROS formation in harbour seal immune cells *in vitro*. Results reported in this paper thus indicate the potential of PBDEs, an important large group of particularly brominated industrial chemicals to interfere with and potentially disrupt the immune system.

Exposure to PBDEs is also associated with enhanced ROS formation in rainbow trout cell lines (Shao et al., 2008), in primary rat hippocampal neurons (He et al., 2008a) and in human neuroblastoma cells (He et al., 2008b). Comparisons with PBDE-related exposure effects on the immune system are difficult due to the limited number of published studies. However, in the present work, the modest enhanced ROS formation, particularly with the increasing concentrations of BDE-47, -99 and -153, is consistent with a study involving the exposure of human granulocytes to similar concentrations of BDE-47 (Reistad and Mariussen, 2005).

PBDEs have been shown to increase the translocation of protein kinase C (Kodavanti and Ward, 2005; Kodavanti et al., 2005; Madia et al., 2004), to cause ^{3}H-arachinodic acid release (Kodavanti and Derr-Yellin, 2002), and to alter cytosolic and mitochondrial calcium homeostasis (Coburn et al., 2008; Dingemans et al., 2008; Kodavanti and Ward, 2005). This latter effect may be of particular relevance, given the importance of calcium in oxidative stress. Calcium is involved in a range of cellular processes, including neurotransmitter release, protein phosphorylation and formation of ROS. It is therefore crucial for the cell to maintain Ca^{2+} homeostasis. The commercial penta-BDE mixture, DE-71, stimulated $[Ca^{2+}]$ elevation in human granulocytes (Reistad and Mariussen, 2005) and this could in part account for the effects of PBDEs on ROS formation. The penta-BDE mixtures were found to induce influx of calcium homeostasis in different cell types ranging from astrocytes to macrophages (Wiegand et al., 2001).

Harbour seal granulocytes play a key role in host defences against invading pathogens and are major effectors of the acute inflammatory reactions. Activation of these cells during an immune response leads to formation of reactive oxygen species used to kill microorganisms. Formation of ROS in granulocytes may be induced by different mechanisms, of which activation of the NADPH oxidase is the most important. The neutrophil NADPH oxidase is a multicomponent membrane-bound enzyme that catalyses NADPH-dependent reduction of oxygen to superoxide radical (O_2^-), which may be converted to hydrogen peroxide (H_2O_2), peroxynitrite ($OONO^-$), hypochlorite (HOCl) and hydroxyl radical (OH) (Babior, 1999). The dichlofluorescein (DCF) assay is an attractive and sensitive method as an overall index for oxidative stress in biological systems. It is reported to detect several types of reactive molecules such as H_2O_2 in the presence of cellular peroxidises, $OONO^-$ and OH, but has no sensitivity toward the superoxide anion radical. It is frequently used to demonstrate activation of respiratory burst in granulocytes (Myhre et al., 2003). The DCF is a primary indicator of intracellular formation of ROS (Caldefie-Chezet et al., 2002).

It is well known that the cell activation process induces intracellular changes, including modification of molecules such as thiols. This study investigated ability of PBDEs to modulate the intracellular balance between ROS production and intracellular thiols. Intracellular thiols such as glutathione play an important role in many physiological processes, including protection from free radical damage and detoxification of chemicals. In this study, the thiol levels decreased slightly with the increasing concentrations of BDE-47, -99 and -153 in adults, pups and cell line of harbour seals. These results indicate that these PBDEs could cause oxidative damage in granulocytes at the levels of enzymes, substrates and products. A change in thiol species such as a reduction of the hepatic glutathione, was also found in female American kestrels (*Falco sparverius*) treated *in ovo* with a mixture of PBDEs (Fernie et al., 2005). Moreover, He et al. (2008a) have shown that

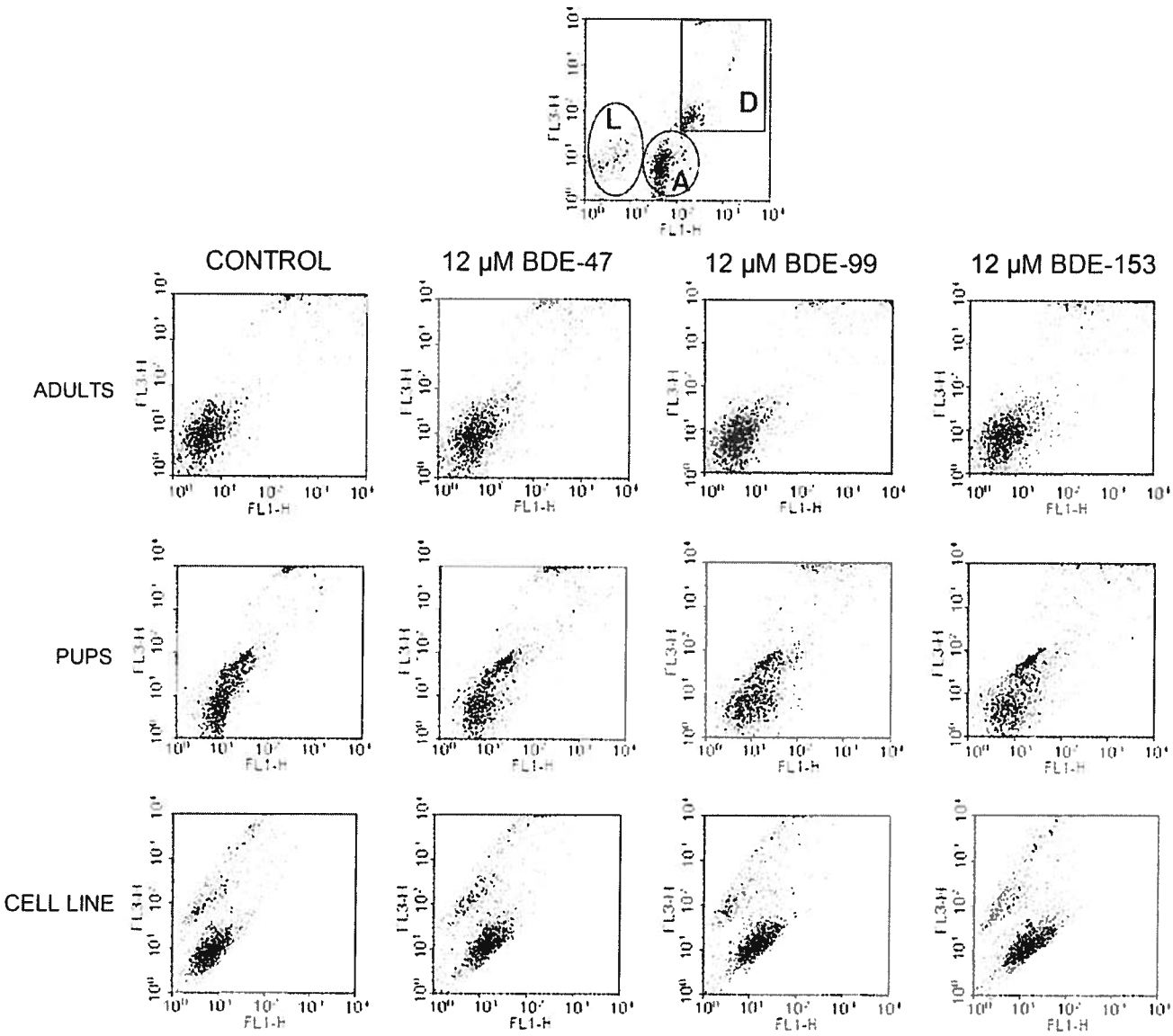

Fig. 4. Effects of positive control (dexamethasone, 1 µM) and BDE-47, -99 and -153 on apoptosis in harbour seal cells and cell line. These dot plots are representative of at least four independent experiments (L: live cells; D: dead cells; A: apoptotic cells).

the glutathione content decreased in primary rat hippocampal neurons exposed to BDE-47 (from 2.06 to 41.2 µM).

In addition to the direct threat to cells and tissue, Koner et al. (1997) showed a possible connection between free radical formation and immune suppression in the rabbit. The structurally-related PCBs have been shown to be linked with decreased phagocytic activity and increased oxidative burst of neutrophil and monocytes in harbour seals (Mos et al., 2006). Altered function of phagocytic cells appears to be largely explained by AhR-independent mechanisms of action and might result from oxidative stress, disturbance of particle uptake, or interference with antioxidant enzymes (Dalton et al., 2002; Narayanan et al., 1998). A PBDE-associated disruption of innate immunity is of concern because these cell types represent a rapid, nonmemory-based immunological defence against many types of invading pathogens, such as bacteria. A previous study reported an association between cellular depletion in lymphoid organs and elevated PCB and PBDE body burdens, supporting the hypothesis of a contaminant-induced immunosuppression in a cetacean species (Beineke et al., 2005). However, animals with higher levels of persistent contaminants had thinner blubber layers and were also in poor body condition, suggesting that they died from illness rather than from an accidental death. In Beineke et al. (2005) it could not be determined if the emaciation was the cause or the result of the pathological findings. The reported association between contaminant levels and lymphoid depletion may be explained by pre-existing diseases causing atrophy of subcutaneous adipose tissue and enrichment of contaminant levels.

Although we did not investigate the effects of BDE -47, -99 and -153 on the expression of biotransformation or on antioxidant genes in harbour seal granulocytes, others have demonstrated that a number of genes critical to cellular protection are down-regulated in the liver of rainbow trout (*Oncorhynchus mykiss*) exposed to BDE-47 *in vivo* (Hook et al., 2006). With the increase in oxidative stress affected by cellular ROS level, the cell membrane, cell proteins and DNA are the targets for ROS attack. ROS are known to be involved in gene regulation (Allen and Tresini, 2000). DNA and chromosomal damage are important events following exposure to environmental agents, especially for genotoxic events. BDE-47 induced DNA damage in human neuroblastoma cells (He et al.,

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2008b) and in rat hippocampal neurons (He et al., 2008a). Barsiene et al. (2006) and Bolognesi et al. (2006) showed in their studies that PBDEs induced micronuclei formation. Helleday et al. (1999) found that PBDEs induced intragenic recombination in mammalian cells, demonstrating genetic effects in mammalian systems. PBDEs do not appear to bind covalently to DNA as suggested by the results from the bacterial reverse mutation assay (Evandri et al., 2003).

Apoptosis is a cellular autonomous death model controlled by genes, which is completely different from cellular necrosis. Some studies have suggested that apoptosis involves the participation, at different levels, of ROS-like hydrogen peroxide, superoxide anion and singlet oxygen (Greenlund et al., 1995; Ishikawa et al., 1999; Wei et al., 2000). The present study investigated the effects of BDE-47, -99 and -153 on apoptosis using flow cytometry and found that the percentage of apoptotic cells was not changed at 12 μM. However, other studies have found that BDE-47 induced cell apoptosis at 41.2 μM in primary rat hippocampal neurons (He et al., 2008a), at 4 μg/mL (8.2 μM) and above in human neuroblastoma cells (He et al., 2008b), while BDE-99 caused apoptosis at 50 μM in human astrocytoma cells (Madia et al., 2004). BDE-71 was reported to cause apoptotic cell death in rat cerebellar granule cells at 5 μM (Reistad et al., 2006) and BDE-209 caused apoptosis in human hepatoma cells Hep G2 at 10 μM (Hu et al., 2007). The mechanisms involved in PBDE-induced apoptosis have not been yet investigated, but are most likely related to oxidative stress.

The present article demonstrates that exposure of harbour seal granulocytes to environmentally relevant PBDEs causes oxidative stress by depleting the level of thiols and/or increasing ROS production and corresponding changes in phagocytic activity and efficiency. The mechanisms of action remain unclear. Observed effects were similar in adults, pups and cell line. Therefore, the 11B7501 cell line could be used as an immune model in the future. Nanomolar concentrations of PBDEs measured in serum of wild harbour seal pups from Bic National Park (Frouin et al., 2009) were too low to induce toxicity to their immune system during their first days of life. However, based on our *in vitro* results, we hypothesize that in pups from other areas with higher concentrations as in the eastern US (Shaw et al., 2008) or in adults, blood concentrations of PBDEs might induce toxic effects on phagocytosis, ROS formation and intracellular thiols levels. Moreover, these effects on the immune function may be exacerbated in wild harbour seals that encounter greater environmental, infectious, physical and nutritional challenges. Together, the results suggest that the immune response of harbour seals exposed to PBDEs could fail to respond appropriately when attacking virus-infected and malignant cells, as well as invading microorganisms. Future research should involve assessing possible synergistic effects on the immune system of wild harbour seals exposed to PBDEs and multiple chemicals.

Acknowledgements

This work was supported by the Canada Research Chair in Environmental Immunotoxicology (M.F.). This research project was funded in part by Fisheries and Oceans, Canada (M.L.). Many thanks to the field crew for their hard work and enthusiasm, most-notably: Steve Trottier, Tiphaine Brett and Gwénaël Beauplet. The expert technical assistance of Marlene Fortier was greatly appreciated. The authors wish to thank the staffs of the zoo at St.-Félicien (Christine Gagnon) and the aquarium du Québec (Dr. Chantal Proulx) for providing blood samples from the marine mammals.

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Please cite this article in press as: Frouin, H., et al. Effects of individual polybrominated diphenyl ether (PBDE) congeners on harbour seal immune cells *in vitro*. Mar. Pollut. Bull. (2009), doi:10.1016/j.marpolbul.2009.09.006

5.3. Effets toxiques de plusieurs polluants sur la lignée cellulaire 11B7501 issue d'un lymphome B de phoque commun (*Phoca vitulina*)

Article 8: *Héloïse Frouin,Marlène Fortier,Michel Fournier. Toxic effects of various pollutants in 11B7501 lymphoma B cell line from harbour seal (Phoca vitulina). Toxicology. (Article soumis le 5 novembre 2009).*

Mon rôle a consisté à faire les analyses, interpréter les données et écrire cet article à titre d'auteur principal. Article soumis le 5 novembre 2009 à la revue Toxicology.

RÉSUMÉ

Les métaux lourds et les hydrocarbures aromatiques polycycliques (HAP) ont souvent été mesurés chez les mammifères marins. Cependant, peu d'information existe sur leurs effets sur la santé du phoque commun (*Phoca vitulina*). Cette espèce est trouvée dans de nombreux habitats incluant les eaux côtiéres contaminées; et peu donc accumuler de fortes concentrations de polluants. Dans cette étude, nous avons estimé les effets immunotoxiques et génotoxiques de plusieurs métaux lourds (arsenic, vanadium, sélénium, fer, argent et chrome) et un HAP (Benzo[a]pyrene ou B[a]P) sur une lignée cellulaire issue d'un lymphome B de phoque commun. Nous avons évalué la viabilité cellulaire, la prolifération lymphocytaire, la phagocytose, l'induction de micronuclei et l'intégrité du cycle cellulaire. Une réduction significative dans la prolifération lymphocytaire a été observée suite à une exposition à 0,05 µM de B[a]P, 5 µM d'arsenic ou sélénium, 50 µM de vanadium, 100 µM d'argent et 200 µM de fer. Le zinc augmente la prolifération à 200 µM. Une diminution de la phagocytose a été observée avec 20 µM d'arsenic, 50 µM de B[a]P ou sélénium, 200 µM de zinc et 500 µM de vanadium. L'induction de micronuclei se produit après l'exposition à 0,2 µM de B[a]P, 100 µM de vanadium et 200 µM d'arsenic ou sélénium. L'exposition à 50 µM d'arsenic diminue la phase G_2/M du cycle cellulaire. Le chrome n'induit aucun effet sur les paramètres immunitaires ou de dommage à l'ADN aux concentrations testées. Les concentrations de métaux lourds (excepté l'argent) et de B[a]P induisant un effet toxique sont dans la gamme de concentrations retrouvées dans le sang des pinnipèdes. Ces résultats supportent l'hypothèse que l'exposition à ces contaminants pourrait nuire à la santé des phoques communs.

Abstract

Altough, heavy metals and polycyclic aromatic hydrocarbons (PAHs) have been reported at high levels in marine mammals, little is know about the toxic effects of some of these contaminants. In this study, we assessed the immunotoxic and genotoxic effects of several heavy metals (arsenic, vanadium, selenium, iron, zinc, silver and chromium) and one PAH (Benzo[a]Pyrene or B[a]P) on a lymphoma B cell line from harbour seal (*Phoca vitulina*). A significant reduction in lymphocyte proliferation was registered following an exposure to 0.05 µM of B[a]P, 5 µM of arsenic or selenium, 50 µM of vanadium, 100 µM of silver and 200 µM of iron. On the contrary, Zinc increased the lymphoproliferative response at 200 µM. Decreased phagocytosis was observed at 20 µM of arsenic, 50 µM of B[a]P or selenium, 200 µM of zinc and 500 µM of vanadium. Micronuclei induction occurred with 0.2 µM of B[a]P, 100 µM of vanadium and with 200 µM of arsenic or selenium. Exposure to 50 µM of arsenic decreased G_2/M phase of the cell cycle. Chromium did not induce any effects on any the parameters of immunity or in damage DNA assays at the concentrations tested. Our results provide a basis for explaining more complex processes which affect the health of those animals subjected to marine contaminants, which can provoke a loss of immunological resistance and play a role in the incidence of infectious diseases or the high prevalence of cancers.

Keywords: heavy metals; PAH; cell line; harbour seal; immunotoxicity; genotoxicity

INTRODUCTION

Environmental contamination might affect the immunocompetence of marine mammals and has been suggested to play a role in recent years in the high incidence of mass mortality events, in unexplained population declines, and in stranding among marine mammal populations (Ross et al. 1996). Furthermore, an imbalance in the immune system caused by pollutants has been suggested to play a role in the incidence of infectious diseases in marine mammals (Bennett et al. 2001). These events have been largely attributed to disease outbreaks caused by established or newly identified members of the genus *Morbillivirus* (Osterhaus and Vedder 1988; Duignan et al. 1995). Moreover, chemical contamination is thought to be involved in the high prevalence of cancer in California Sea lions (*Zalophus californianus*) and in St. Lawrence beluga whales (*Delphinapterus leucas*) (De Guise et al. 1994; Martineau et al. 1994; Gulland et al. 1996). DNA damage is known to be involved in the development of cancer and can

be a risk factor for teratogenesis and for other pathological conditions (Tucker and Preston 1996).

The harbour seal (*Phoca vitulina*) is an ideal model organism for investigating contaminant-induced immune alterations in marine mammals (Ross 2000). Seals eat fish, they are long-lived, they maintain large adipose deposits, and they occupy high trophic levels in the marine food chain; consequently they accumulate relatively high levels of lipophilic contaminants (Ross et al. 1996). Harbour seals are of interest because they are non-migratory and they live in a variety of habitats including coastal areas that can be either uncontaminated or contaminated. Various studies have documented high levels of heavy metals in phocids in different parts of the world (Yamamoto et al. 1987; Fossi et al. 1997; Watanabe et al. 1998; Saeki et al. 1999; Kutoba et al. 2001; Saeki et al. 2001; Veinott and Sjare 2006; Brookens et al. 2007). The available literature reports mainly concentrations of contaminants in the liver and kidneys. Invertebrates living in sediments contaminated by polycyclic aromatic hydrocarbons (PAHs) accumulate these compounds, in contrast to vertebrates (Ferguson and Chandler, 1998). However, Marsili et al. (2001) detected concentrations of PAHs in cetaceans from Mediterranean such as whales (1.97 µg/g fresh weight) and dolphins (29.5 µg/g f.w.) and in sea lions from Argentina (7.46 µg/g f.w.).

Heavy metals and PAHs are known to produce toxic effects on animals. Altered immune parameters associated with in vitro exposure to PAHs (notably benzo[a]pyrene) have been reported in harbour seal and include depressed T-lymphocyte proliferative response to mitogen (Neale et al. 2002). The immunosuppressive influence, notably of metal pollutants on parameters of non-specific cellular immunity such as phagocytosis, mitogen-induced proliferation and natural killer cytotoxicity was investigated in several marine mammal species but only for a few selected metals (De Guise et al. 1996; Pillet et al. 2000; Kakuschke et al. 2005; Kakuschke et al. 2006; Camara Pellisso et al. 2008).

Certain environmental contaminants found in marine mammals, such as PAHs and heavy metals, have been shown to cause DNA damage (Gauthier et al. 1998; Gauthier et al. 2003). Micronuclei are chromosomal fragments or whole chromosomes that are not incorporated into daughter nuclei during mitosis because of chromosomal breakage or whole chromosome loss (i.e. aneuploidy), respectively. Micronuclei can thus be used as a biomarker of fundamental importance to measure the molecular mechanisms involved in toxicity since it can show the real interference with the mitotic apparatus.

In this pilot study, we used a model cell line (11B7501 lymphoma B cell line) to determine the action of seven metals (Ag, As, Cr, Fe, Se, V, Zn) and benzo[a]pyrene (B[a[P) on the function of immune cells. A previous study demonstrated that the 11B7501 cell line could be used as an immune model (Frouin et al. 2009). These pollutants were chosen because they were detected in phocid tissues and the lack of information about their effects in marine mammals. B[a]P is a model PAH compound which has been widely used as an index of PAH contamination and as a positive control in in vitro genotoxicity and immunotoxicity studies (Gauthier et al. 1999; Neale et al. 2002).

MATERIAL AND METHODS

Cultured cell line

The 11B7501 B lymphoma cell line was purchased from the American Type Culture Collection (CRL-1940). It was developed by P. Marx et al. in 1985 from the peripheral blood mononuclear cells derived from a male harbour seal with malignant leukemia lymphoma. Cells were grown in complete medium [RPMI-1640 with 10% heat-inactivated foetal bovine serum (FBS), 1% Pen-Strept and 10 mM Hepes] at 37℃, 5% CO_2 in a humid atmosphere. For routine culturing, the cells were maintained at 10^5 - 10^6 cells/ml. The scattergram of the flow cytometric profiles of these cells (Fig. 1) indicates that two subpopulations, B/macrophage cells and lymphocytes, were present.

Chemical preparation

Vanadium (V) oxytrichloride ($VOCl_3$), arsenic (III) chloride ($AsCl_3$), silver chloride (AgCl), chromium (III) chloride ($CrCl_3$), iron (III) chloride ($FeCl_3$), zinc chloride ($ZnCl_2$), selenium tetrachloride ($SeCl_4$) and benzo[a]pyrene (B[a]P) were obtained from Sigma-Aldrich Chemical Co. Dimethylsulfoxide (DMSO) and ethanol (ETOH) were purchased from Sigma Chemical Co. Various concentrations of $VOCl_3$, AgCl and $ZnCl_2$ were dissolved in ETOH. Similarly, $CrCl_3$ and B[a]P were dissolved in DMSO. $AsCl_3$, $FeCl_3$ and $SeCl_4$ were dissolved in distilled water. The final concentration of DMSO or ETOH did not exceed 0.5%. Equivalent volumes of DMSO or ETOH were added to control cells.

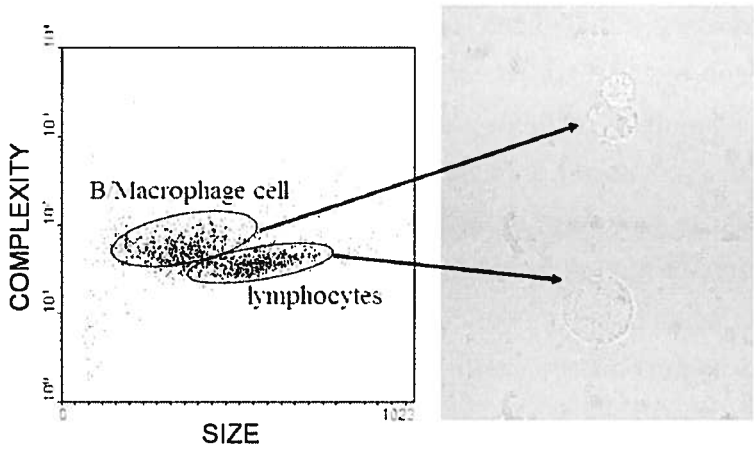


Figure 1. Scattergram of the flow cytometric profile of 11B7501 lymphoma B cells. In order to identify different cell subpopulations on the basis of the cell size and cytoplasm complexity, the flow cytometric method was used in the present study. Two populations were isolated by analytical gates, lymphocytes and B/macrophage cells. All images original magnification ×1000.

In vitro exposures

For viability and phagocytosis, cells adjusted to a concentration of 10^6 cells/ml were exposed at 37℃ in a humidified 5% CO_2 atmosphere for up to 24 h to 5, 10, 20 and 50 µM of $AsCl_3$ or B[a]P and to 50, 100, 200 and 500 µM of $VOCl_3$, AgCl, $CrCl_3$, $FeCl_3$, $ZnCl_2$ and $SeCl_4$ in complete medium.

For viability and lymphocyte proliferation, 5×10^6 cells/ml were exposed at 37℃ in a humidified 5% CO_2 atmosphere for up to 66 h to 0.05, 0.1, 0.5, 1, 2 and 5 µM of B[a]P and 5, 10, 50, 100, 200 and 500 µM of $AsCl_3$, $VOCl_3$, AgCl, $CrCl_3$, $FeCl_3$, $ZnCl_2$ and $SeCl_4$ in complete medium.

For viability, micronucleus assay and cell cycle 5×10^5 cells/ml were exposed at 37℃ in a humidified 5% CO_2 atmosphere for up to 24 h to 0.05, 0.1, 0.2, and 0.5 µM of B[a]P and 50, 100, 200 and 500 µM of $AsCl_3$, $VOCl_3$, AgCl, $CrCl_3$, $FeCl_3$, $ZnCl_2$ and $SeCl_4$ in complete medium. After 24h, cells were resuspended in complete medium without contaminants during 72 h.

Viability

Viability of cells after exposure was evaluated by flow cytometry using propidium iodide (PI) (100 µg/ml) as described by Brousseau et al. (1999). PI enters in all cells but is actively excreted only by living cells. Samples were analysed in a FACScalibur flow cytometer (Becton Dickinson, San José, USA) and the fluorescence of 5 000 events was read in FL3. Data are expressed as a percent of viable cells.

Phagocytosis

Following a 24-h incubation, phagocytic activity was measured using the protocol of Brousseau et al. (1999). We used fluorescent latex beads that were ingested by cells. Samples were analysed with a FACScalibur flow cytometer (Becton Dickinson, San José, USA) equipped with a 488-nm argon laser. For each sample, the fluorescence of 10,000 events was recorded. Results were analysed with the Cell Quest Pro software (Becton Dickinson) to determine the percentage of cells that engulfed one bead and more (phagocytic activity) or three beads and more (phagocytic efficiency). The absence of fluorescent response observed in lymphocytes corresponds to their inability to perform phagocytosis and represents an appropriate negative control. The results were expressed as the percentage of phagocytosis.

Measurement of lymphocyte proliferation

Increased DNA synthesis due to cell proliferation was measured as the incorporation of ^{3}H-thymidine. Briefly, 100 µl of cells were plated with 100 µl complete medium and 10 µl of pollutants for 48 h at 37℃ and 5% CO_2 in 96-well round-bottom plates. Samples were tested in triplicate. After a 48-h incubation, 1 µCi of ^{3}H-thymidine was added in each well and plates were incubated for a further 18 h. The cells were harvested onto glass fiber filter with a semi-automatic cell harvester and the amount of radioactivity incorporated was measured with a Trilux counter. The raw data were expressed as counts per minute (CPM), triplicates were averaged, and the results were presented as the percentage of the control.

Micronucleus test and cell cycle

A suspension of nuclei and micronuclei was prepared for flow cytometric analysis using the two step procedure developed by Nusse et al. (1984). Harvested cells (5×10^5 cells/ml) were centrifuged at 300g for 5 min. The supernatant was removed and the cell pellet was resuspended in solution I (584 mg/l NaCl, 1000 mg/l Na citrate, 10 mg/l Rnase, 0.3 ml/l Nonidet P-40 substitute, 25 mg/l ethidium bromide) then in solution II (1.5 % citric acid, 0.25 M sucrose, 40 mg/l ethidium bromide).

Micronuclei and cell cycle data acquisition and analysis were performed with a FACSCalibur flow cytometer. Pulse height signals of forward light scatter (FSC) and side light scatter (SSC) were collected, and ethidium bromine emission measured using fluorescence channel FL-2. Micronuclei (MN) and nuclei (N) were analysed based on the

gating strategies and size criteria established in previously published methods (Roman et al. 1998; Sánchez et al. 2000; Suggitt et al. 2003). G1-phase nuclei were stored around channel 2000 and micronuclei were discriminated from unspecific debris using gates set a 0.5 and 50% of the DNA content of G1-phase nuclei (the left and right limits of gate were channel 10 and 10,000 respectively). Nuclei using R1 and R3 gates (from channel 2000 to 10,000), and MN containing a 1-10% of DNA of G1 phase nuclei were defined using R2 and R4 gates. 10,000 events of each sample were acquired to estimate MN frequencies. The frequency of MN was calculated according to the following formula: FMN = MN/N = R2*R4/R1*R3, where FMN represents MN frequencies, MN/N is the quotient between the number of MN and the number of N in each sample and R1*R3 and R2*R4 represent the number of repeated events in respective gates both in FSC and SSC dot plots (Fig. 2).

For the determination of the cell cycle phases, a peak area of FL2-H was recorded on a log scale. The quantitative cell cycle and DNA histogram peak analysis were performed using the Cell Quest Pro software (Becton Dickinson). The percentages of cells in G0/G1, S and G2/M cell cycle phases were calculated (Fig. 2). To evaluate cell cycle alterations, 5000 events were acquired.

Statistical analyses

Differences between controls and treated groups were evaluated by one-way ANOVA followed by Dunnett's two-sided post hoc test. The calculations were performed using STATISTICA for windows (version 7.0, StatSoft Inc, 1995).

RESULTS

Cell viability

The sensitivity of the 11B7501 cell line to different concentrations of $VOCl_3$, $AsCl_3$, AgCl, $CrCl_3$, $FeCl_3$, $ZnCl_2$, $SeCl_4$ and B[a]P after 66 h or 24 h of exposure are shown in Tables 1 and 2, respectively. Viability was significantly decreased after 24 h of incubation with 50 μM of $SeCl_4$ ($p < 0.01$) or with 200 μM of $ZnCl_2$ ($p < 0.05$). Cell viability showed no toxic effects for $AsCl_3$, AgCl, $CrCl_3$, $VOCl_3$, $FeCl_3$ and B[a]P during 24 h of incubation. All pollutants except $CrCl_3$ caused a significant decrease in cell viability during 66 h of exposure. Viability was significantly decreased after 66 h of incubation with 100 μM of

$VOCl_3$ ($p < 0.05$), 200 µM of $ZnCl_2$ ($p < 0.01$) and 500 µM of $FeCl_3$, $SeCl_4$ ($p < 0.01$) or AgCl ($p < 0.05$).

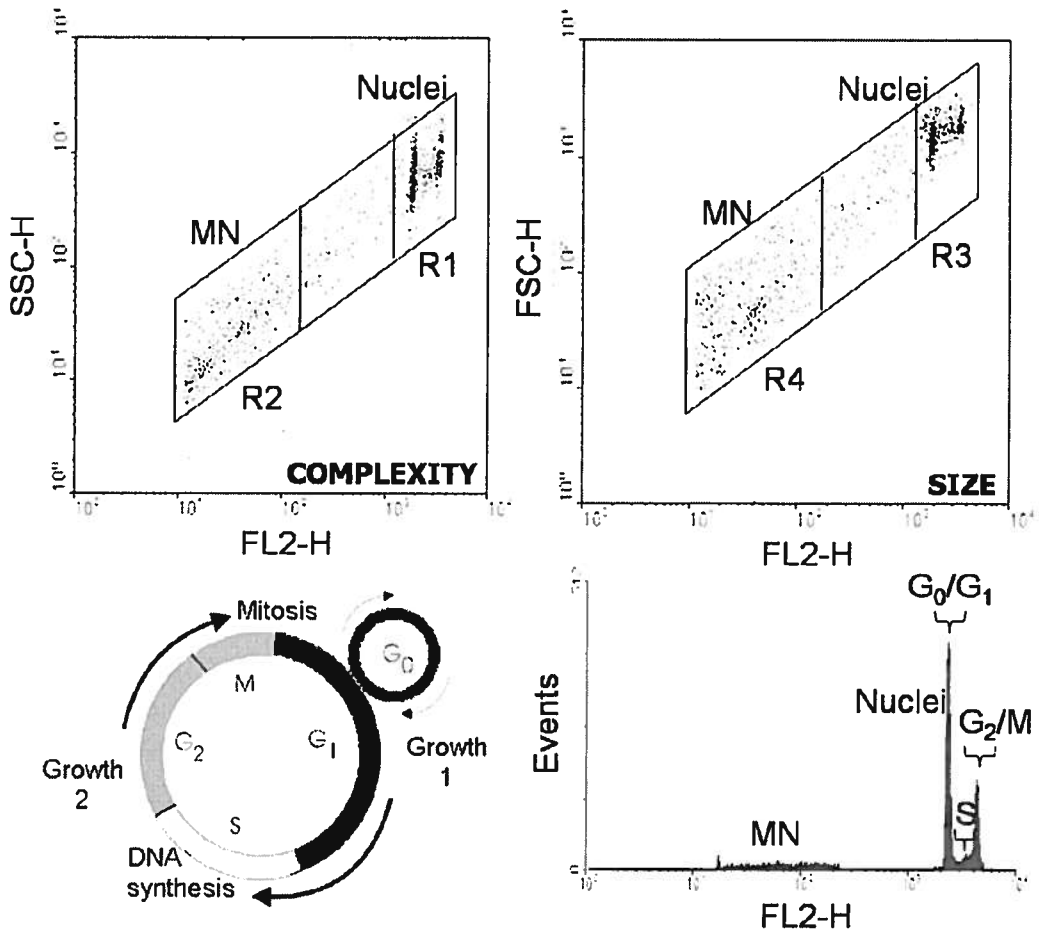


Figure 2. Dot plots of complexity and size obtained from the events in a suspension of micronuclei and nuclei from vehicle. Gates R1 and R3 were used to calculate the frequency of nuclei; gates R2 and R4 were used to calculate the frequency of micronuclei. Histogram was used to quantitatively evaluate cell cycle distribution.

Phagocytosis

The phagocytic activity was significantly ($p < 0.05$) reduced with the lowest concentration of $SeCl_4$ (50 µM) and with the highest concentration of $VOCl_3$ (500 µM) and B[a]P (50 µM) (Fig. 4). The phagocytic efficiency was significantly ($p < 0.05$) reduced by the lowest concentration of $SeCl_4$ (50 µM) and the highest concentration of $VOCl_3$ (500 µM) and B[a]P (50 µM). The highest concentrations (20 and 50 µM) of $AsCl_3$ also significantly decreased the phagocytic activity ($p < 0.05$) and the phagocytic efficiency ($p < 0.01$). No effect was observed with $CrCl_3$, $FeCl_3$, AgCl and $ZnCl_2$ at the concentrations tested.

Proliferation

$SeCl_4$, $AsCl_3$, $VOCl_3$ and B[a]P were the pollutants which most affected the proliferation of cells (Fig. 3). The proliferation was significantly reduced by the lowest concentration of $SeCl_4$, $AsCl_3$ (5 μM) ($p < 0.01$) and B[a]P (0.05 μM) ($p < 0.05$). $VOCl_3$ significantly reduced ($p < 0.05$) the proliferation at 10 μM. AgCl and $FeCl_3$ significantly decreased the proliferation of cells at 100 μM ($p < 0.05$) and 200 μM ($p < 0.01$), respectively. $ZnCl_2$, at 200 μM, also affected proliferation but caused a significant ($p < 0.01$) increase by 108 % followed by a decrease not significant at 500 μM by 36 %. No effect was observed with $CrCl_3$ at the concentrations tested.

Micronucleus test

Results for MN induction in cell line treated with pollutants (Fig. 5) demonstrated that exposure with $VOCl_3$, $AsCl_3$, $SeCl_4$ and B[a]P resulted in a dose-dependent increase in MN formation. At 0.2 μM benzo[a]pyrene induced significantly ($p < 0.01$) MN formation. $VOCl_3$ induced significantly ($p < 0.05$) MN formation at 100 μM and $AsCl_3$ and $SeCl_4$ increased significantly ($p < 0.01$) MN induction at 200 μM.

Cell cycle

The proportion of cells in the G_0/G_1, S and G_2/M phases (Fig. 6) showed that the progression of cells through the cell cycle was affected in a concentration-dependent manner only by $AsCl_3$, which reduced significantly ($p < 0.05$) proportions of G_2/M cells for the lowest concentration (50 μM). The cell cycle was unaffected by the other contaminants.

Table 1. Viability of harbour seal cells following exposure to metals and B[a]P during mitogenic assay (66 h of exposure) (n =4). Results (mean ± standard error) are expressed as a % of the control response (unexposed cells). * or ** mean significantly $p < 0.05$ or $p < 0.01$ different from control (100 %), respectively.

Pollutants	**5 µM**	**10 µM**	**50 µM**	**100 µM**	**200 µM**	**500 µM**
$VOCl_3$	99.07 ± 0.37	99.69 ± 0.48	98.87 ± 0.22	95.37 ± 1.32*	92.40 ± 2.53*	94.84 ± 0.23
$FeCl_3$	99.28 ± 1.34	100.61 ± 1.00	100.44 ± 0.84	97.94 ± 1.81	99.21 ± 1.63	91.21 ± 2.03**
$AsCl_3$	98.34 ± 0.35	98.68 ± 1.69	93.07 ± 4.74	90.07 ± 3.61	93.13 ± 2.88	91.21 ± 0.17
$SeCl_4$	95.67 ± 0.75	91.60 ± 0.16	90.04 ± 3.47	89.59 ± 4.62	91.34 ± 5.65	77.55 ± 2.93**
AgCl	99.07 ± 0.90	100.16 ± 0.85	100.22 ± 0.78	100.31 ± 0.87	95.63 ± 4.04	83.44 ± 7.21*
$ZnCl_2$	99.28 ± 0.58	99.34 ± 0.14	99.81 ± 0.55	97.85 ± 0.81	91.63 ± 0.67**	89.40 ± 3.37**
$CrCl_3$	99.70 ± 0.41	100 ± 0.40	99.91 ± 0.14	98.23 ± 0.72	99.54 ± 0.93	99.87 ± 0.74
Pollutants	**0.05 µM**	**0.1 µM**	**0.5 µM**	**1 µM**	**2 µM**	**5 µM**
B[a]P	98.48 ± 0.58	98.73 ± 0.85	99.08 ± 0.29	97.87 ± 0.03	96.59 ± 2.15	96.76 ± 0.72

Table 2. Viability of harbour seal cells following exposure to metals and B[a]P during phagocytic assay (24 h of exposure) (n =4). Results (mean ± standard error) are expressed as a % of the control response (unexposed cells). * or ** mean significantly $p < 0.05$ or $p < 0.01$ different from control [0], respectively.

Pollutants	**[0]**	**50 µM**	**100 µM**	**200 µM**	**500 µM**
$VOCl_3$	77.20 ± 4.50	70.95 ± 5.34	68.37 ± 6.13	58.25 ± 14.65	56.93 ± 6.79
$FeCl_3$	77.20 ± 4.50	72.95 ± 3.85	68.84 ± 9.80	67.10 ± 2.24	63.74 ± 3.42
$SeCl_4$	77.20 ± 4.50	52.22 ± 9.40*	51.67 ± 0.13**	48.78 ± 3.07**	44.71 ± 0.07**
AgCl	77.20 ± 4.50	76.24 ± 3.52	78.20 ± 3.14	71.36 ± 9.24	79.32 ± 5.49
$ZnCl_2$	77.20 ± 4.50	68.88 ± 1.42	69.53 ± 1.55	63.21 ± 0.73**	58.63 ± 2.59**
$CrCl_3$	77.20 ± 4.50	74.79 ± 3.35	70.34 ± 0.02	76.70 ± 4.11	75.57 ± 3.22
Pollutants	**[0]**	**5 µM**	**10 µM**	**20 µM**	**50 µM**
$AsCl_3$	77.20 ± 4.50	77.28 ± 3.69	77.87 ± 2.22	78.22 ± 2.02	74.21 ± 2.74
B[a]P	77.20 ± 4.50	76.72 ± 2.27	76.52 ± 4.22	73.56 ± 5.38	71.97 ± 4.83

Figure 3. Proliferation assay of harbour seal cells following the treatment with metals and B[a]P (n = 5). Results are given as mean ± standard error. One way ANOVA followed by Dunnett.s two-sided post hoc test was performed to indicate statistical significant differences between exposure groups and control (* $p < 0.05$; ** $p < 0.01$). The level control (100%) represents results from cell line cultures without pollutants.

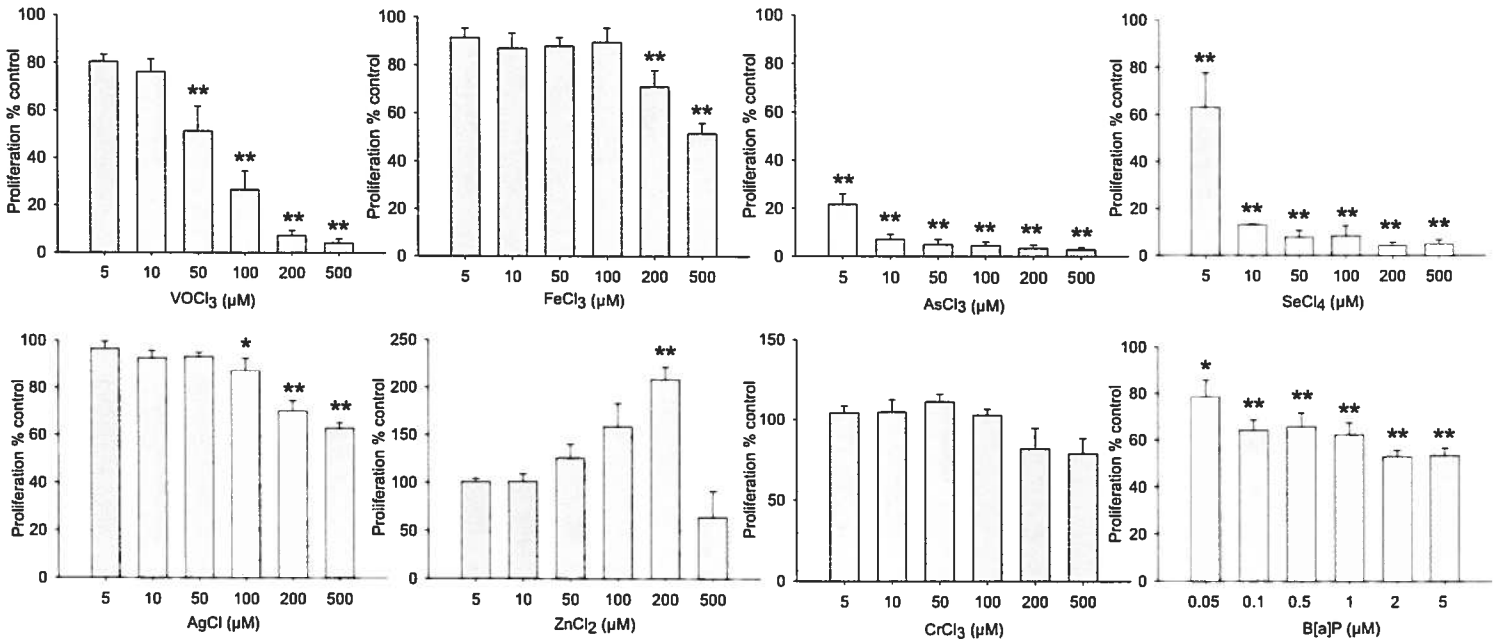


Figure 4. Phagocytic assay of harbour seal cells following the treatment with metals and B[a]P (n = 5). Results are given as mean ± standard error. One way ANOVA followed by Dunnett.s two-sided post hoc test was performed to indicate statistical significant differences between exposure groups and control (* $p < 0.05$; ** $p < 0.01$).

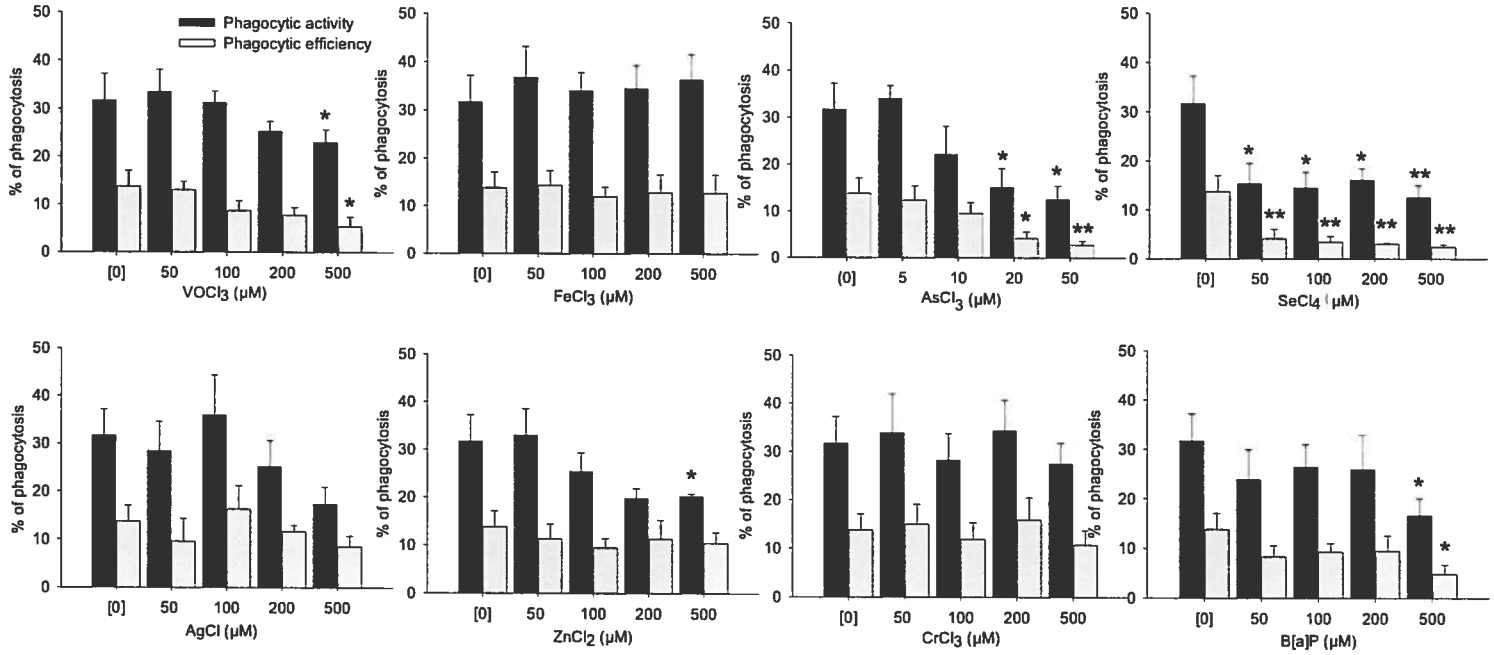


Figure 5. Micronucleus data are graphed for each of eight pollutants studied. Mean micronucleus responses (fold-increase values) are shown for flow cytometry-based assessments (n = 5). Asteriks indicate statistical significant differences between exposure groups and control (* p < 0.05; ** p < 0.01). Doses that significantly decreased viability are indicated by a bracket and the word cytotoxic.

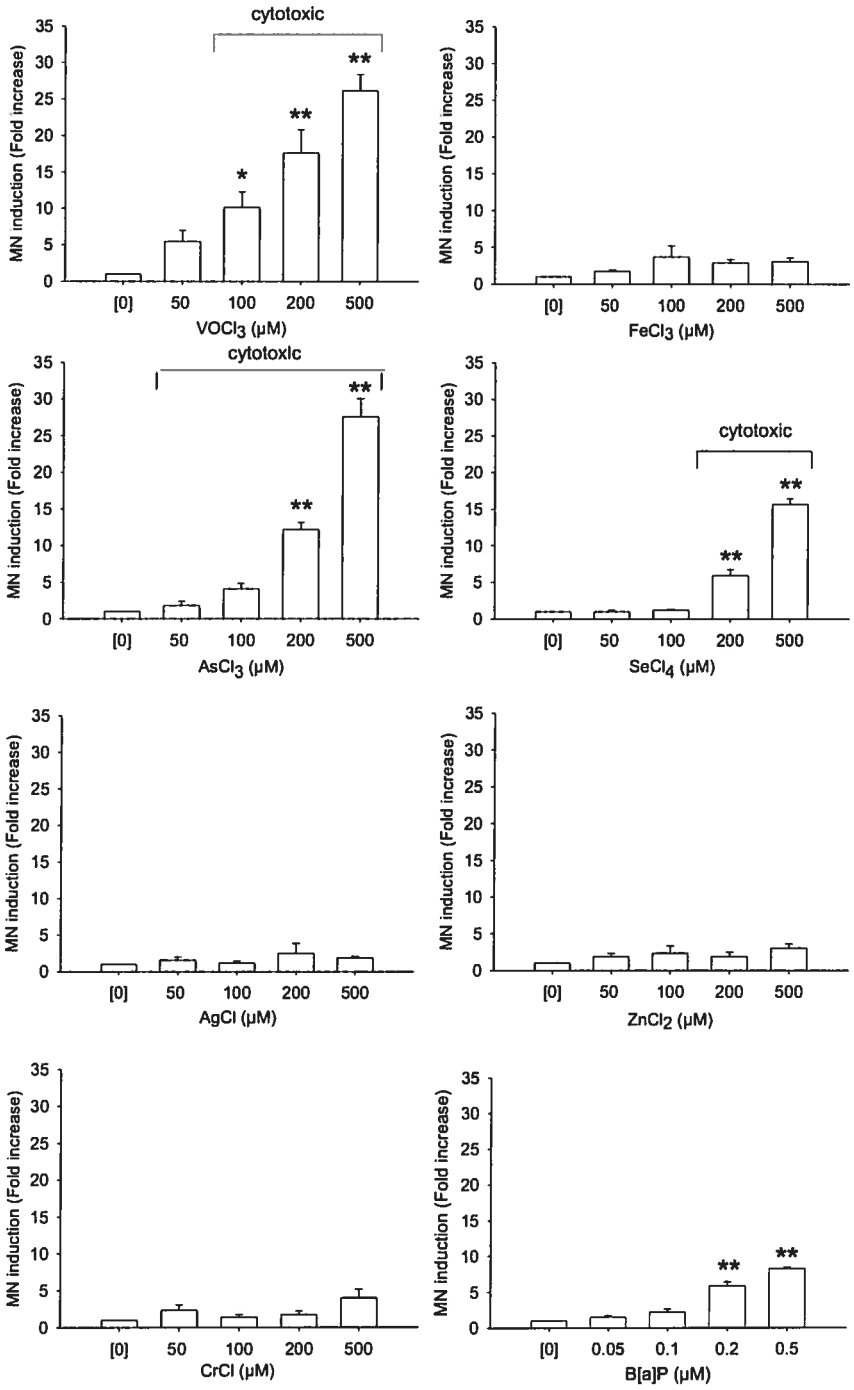
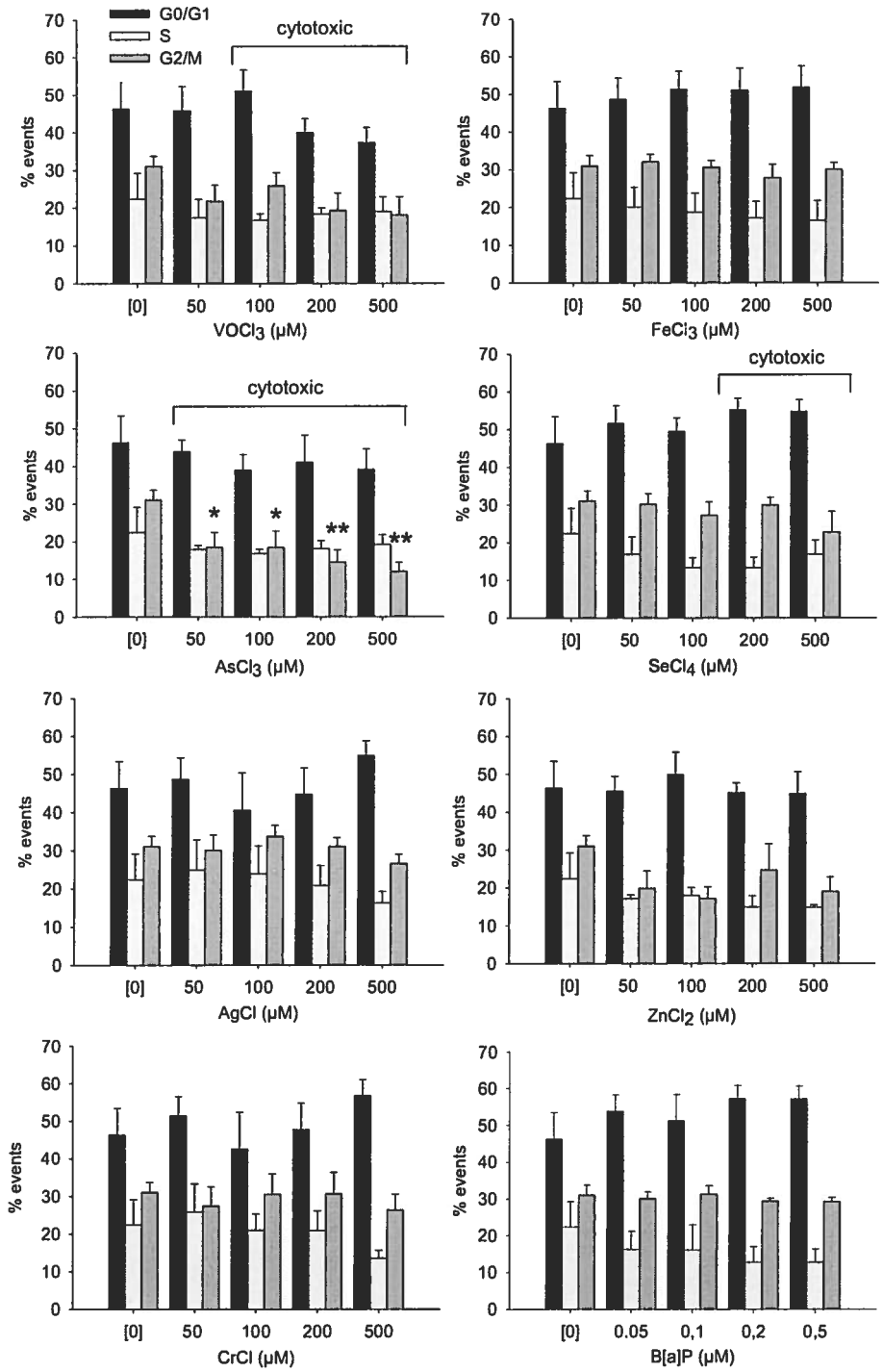


Figure 6. Cell cycle distribution data are graphed for each of eight pollutants studied. Mean percentage of events are shown for flow cytometry-based assessments (n = 5). Doses that significantly decreased viability are indicated by a bracket and the word cytotoxic. Asteriks indicate statistical significant differences between exposure groups and control (* $p < 0.05$; ** $p < 0.01$).



DISCUSSION

Results presented in this study clearly demonstrate that some of heavy metals and B[a]P, a PAH, are able to decrease phagocytosis and lymphoblastic proliferation as well as induce micronuclei formation and modifications in the cell cycle in a harbour seal cell line. These results thus show that heavy metals and PAHs produce alterations in the immune function and increase the incidence of infectious diseases in marine mammals.

Macrophages provide the most important host defence mechanism against invading microorganisms by phagocytosis followed by intracellular killing and digestion of microorganisms during various microbial infections. In this study, $AsCl_3$ decreased phagocytic activity and efficiency at 20 µM. Previous studies demonstrated than an *in vivo* $NaAsO_2$ exposure suppressed phagocytosis by mouse macrophages (Sengupta and Bishayi 2002; Bishayi and Sengupta 2003). The inhibitory effect on phagocytosis as demonstrated in our study (after an exposure to 500 µM of $VOCl_3$) has also been reported with vanadium compounds in *in vitro* cultured murine, rabbit, and bovine pulmonary alveolar macrophages (Waters et al. 1974; Fisher et al. 1978; Wei and Misra 1982). In these reports the decreased phagocytic activity and intracellular killing were related to reduced surface opsonin receptor expression/binding activity and lysosomal enzyme release and activity (Vaddi and Wei 1991a; Vaddi and Wei 1991b). Previous studies have shown the positive effects of selenium supplementation on human health (Ferencik and Ebringer 2003). However, *in vitro* exposure of harbour seal cell line with $SeCl_4$ inhibited phagocytosis by these cells at the lowest concentration (50 µM) which could be linked to cytotoxicity. A similar inhibition in goat and bovine leukocytes has been reported following *in vitro* supplementation of selenium (Aziz et al. 1984; Ndiweni and Finch 1996). In our study, $ZnCl_2$ decreased phagocytic activity after an exposure to 500 µM. However, Pillet et al. (2000) demonstrated that $ZnCl_2$ did not cause a significant decrease of phagocytosis in harbour seal or grey seal (*Halichoerus grypus*) leukocytes. In human monocytes, the phagocytosis of fluorescent microspheres was down-regulated in response to B[a]P (10 µM) (Van Grevenynghe et al. 2003). However, in our study, B[a]P decreased phagocytic activity and efficiency only at 50 µM.

The anti-proliferative effects of several heavy metals and PAHs are well documented in marine mammals (De Guise et al., 1996; Neale et al., 2002; Camara Pellisso et al., 2008). In our study, $AsCl_3$ inhibited the stimulation and proliferation of lymphocytes at concentrations as low as 5 µM. Previous works demonstrated that *in vitro*, both trivalent and pentavalent arsenicals inhibit murine (Yoshida et al. 1986) and

bovine (Burns et al. 1997) phytohemaglutinin (PHA)-stimulated lymphoproliferation at concentrations of ≥ 3 μM. Previous studies using a model of murine osteoblast-like cells in culture have shown that vanadium compounds exert a biphasic effect on cell growth: low micromolar concentrations stimulated cell proliferation while high concentrations have the opposite effect (Cortizo and Etcheverry 1995; Barrio et al. 1997; Etcheverry et al. 1997; Salice et al. 1999). Our data are in agreement with the observation of Roldan and Altamirano (1990), who showed that concentrations between 2-6 μg/ml (11-33 μM) of vanadium produced a significant decrease in mitotic index in human lymphocytes. In the present study, $SeCl_4$ inhibited lymphocyte proliferation at 5 μM. This result agrees with a previous work, which demonstrated that inhibition of the proliferation was noted at selenium concentrations as low as 1 μM on human lymphocytes (Petrie et al. 1989). In human lymphocytes, Zn ions induced a maximum increase in lymphocyte DNA synthesis at 100 μM Zn^{2+}, decreased at higher concentrations (200 μM and 300 μM) and inhibited DNA synthesis at 500 μM (Berger and Skinner 1974). In the present study a significant increase in lymphocyte proliferation occurred at 100 μM of $ZnCl_2$ but not as effectively as at 200 μM and the lymphocyte proliferation decreased at 500 μM. Exposure of lymphocytes to increasing concentrations of iron in the form of ferric citrate ($C_6H_8O_7Fe$) showed iron to inhibit mitogen responses to PHA and Con A stimulation at 300 μM (Bryan and Leech 1983). In the present work a significant decrease of the lymphocyte proliferation was observed at 200 μM of $FeCl_3$. Silver salts have been shown to exert an inhibitory effect on proliferation and differentiation of several cell lines such as human lymphocytes (Hussain et al. 1992) with a IC_{50} at 30 μM. In the present study, AgCl inhibited lymphocyte proliferation of harbour seal cell line at 100 μM. The B[a]P exposure showing depressive effects *in vitro* on lymphocyte proliferation in the present study (0.05 μM) is equivalent to 12.6 ng/mL, a concentration close to that found in the blood of Southern sea lion (Table 3). Neale et al. (2002) demonstrated that in harbour seal, in vitro B[a]P exposure to 0.1 and 1.0 μM decreased the T-cell proliferative response, but a significant suppressive effect on T cell mitogenesis was observed only after an exposure to 10 μM.

MN induction in harbour seal cell line occurred at 200 μM of $AsCl_3$. Previously, arsenite had been shown to cause a significant induction of MN in human lymphocytes *in vitro* at 0.5 μM (Schaumloffel and Gebel 1998) as well as at 2 μM (Colognato et al. 2007) and in chinese hamster ovary cells at 10 μM (Gurr et al. 1998). However arsenate, another inorganic arsenic, induced a significant increase of MN frequency in peripheral

human lymphocytes at the dose 500 μM (Colognato et al. 2007). Vanadium has been shown to cause a significant increase of MN *in vitro* in human lymphocytes at 80 μM (Migliore et al. 1995) and in Chinese hamster V79 cells at 1 μg/ml (5.5 μM) (Zhong et al. 1994). With vanadium the number of binucleated cells significantly decreased from 89% in the control to 23-32% at the range of concentration tested (3-12 μg/ml) (Zhong et al. (1994)). In our study, MN induction in harbour seal cell line occurred only at 100 μM of $VOCl_3$. Khalil (1989) has shown that increased doses of sodium selenite (Na_2SeO_3) added to human lymphocytes stimulated with PHA increased the risk of chromosomal aberrations. In cultures containing 6 ng Se/ml (0.076 μM) as selenite, 13% of cells were aberrant and at 600 ng/ml (7.6 μM) the value rose to 40%. MN induction in harbour seal cell line occured only after an exposure to 200 μM of $SeCl_4$. Tetravalent selenium (H_2SeO_3) induced micronuclei formation in mice exposed *in vivo* to 5 mg/kg (Itoh and Shimada 1996). It has been reported that selenite at concentrations of 480-800 μg Se/litre (6-10 μM selenite) is cytotoxic to human lymphocytes causing an increased frequency of MN and inhibited cell division (Berces et al. 1993; Biswas et al. 2000; Abul-Hassan et al. 2004). This may be due to increased oxidative stress and breaks in DNA strand in cells induced by selenite (Biswas et al. 2000; Zhou et al. 2003; Kim et al. 2004). The induction of micronuclei at concentrations as low as 0.2 μM of B[a]P agrees with results observed in the article of Warshawssky et al. (1995), which demonstrated that B[a]P at 0.1 μg/ml (0.4 μM) induced micronuclei in human lymphocytes. Concentrations of 0.5, 2 and 5 μg/ml (2, 8 and 20 μM) induced a 2.2-, 2.7- and 5.5-fold mean increase in micronuclei induction in beluga whale skin fibroblasts (Gauthier et al. 2003).

The cell cycle consists of four distinct phases: G_1 phase, S phase (synthesis), G_2 phase (interphase) and M phase (mitosis) and when cells have temporarily or reversibly stopped dividing they have entered a state of quiescence called G_0 phase. A previous study reported that concentrations of inorganic arsenic between 10^{-9} and 10^{-7} M inhibited human lymphocyte stimulation and proliferation during G_0 (Gonsebatt et al. 1992). However, in the present study the most sensitive stage with $AsCl_3$ was during G_2/M. Galicia et al. (2003) demonstrated that in presence of arsenite, the synthesis and secretion of lymphokines is delayed and that a delay in the cell cycle also occurred, since a higher number of cells were found in the G_0/G_1 phase and cell percentage in S and G_2/M phase was reduced. These results are in agreement with those previously reported in different cell lines (Park et al. 2001; Wang et al. 2001) and those observed in the present study. The transition form G_1 into the replicative phases of the cell cycle is

mediated by IL-2 (Cantrell and Smith 1984; Smith 1988); the magnitude and extent of lymphocyte proliferation are influenced by the concentration of IL-2 produced and the period during which it is available (Cantrell and Smith 1984). Galicia et al. (2003) suggested than since no G1 progression factor is present (IL-2) during the first time points of the $NaAsO_2$-treated cells, the T lymphocytes are blocked in the G_1 phase. However, at later time points, IL-2 is synthesized and secreted, although at lower levels, and the T cells can progress from the G_1 to the S phase. In the present study, only arsenic induced a significant effect on the cell cycle. But, the cell cycle could be also delayed by vanadium like demonstrated in Zhong et al. (1994). Both vanadate (Hamaguchi et al. 1995) and pervanadate (Faure et al. 1995) interrupted the cell cycle at the G_2/M phase. A previous study suggested that cycling human T-lymphocytes were sensitive to B[a]P at concentrations as low as 0.01 μM and that expression or manifestation of toxicity required that cells enter or traverse S phase, which is the DNA replication phase of the cell cycle (Mudzinski 1993). This study also suggested that T-cell processes through the late G_1 phase, which require cooperation with macrophages, are relatively refractory to concentrations of B[a]P as high as 1 μM (Mudzinski 1993). These results disagree with the observations in the present study, which demonstrated that B[a]P neither increased G_0/G_1 nor decreased G_2/M phase significantly.

Heavy metals and PAHs can produce alterations in the immune function (Bernier et al. 1995; Reynaud and Deschaux 2006) and the DNA (Sanchez-Galan et al. 1999; White 2002). In the present work, an *in vitro* approach has been developed to test the effects of seven heavy metals and one PAH on the immune and genetic responses from harbour seals. Using these assays we can confirm that arsenic, vanadium, selenium, B[a]P are the most toxic pollutants for harbour seal immune cells, followed by zinc, iron and silver, whereas chromium does not induce any alteration as demonstrated previously *in vitro* in bottlenose dolphin leukocytes (Camara Pellisso et al. 2008). The immunotoxic and genotoxic concentrations reported in this study are within the ranges measured in blood of pinnipeds, excepted for the silver (reviewed in Table 3). Generally, high concentrations in blood indicate a recent intake, e.g., with food. Blood values reflect contaminant concentration in the circulating blood stream and define the actual body burden, dependent on the half-life of the pollutant under study. The reduction of some functional activities of the harbour seal immune system may cause a significant weakness capable of altering host resistance to disease in free-ranging pinnipeds, as

has been suggested in the case of other marine mammal species exposed to different pollutants (Bennett et al. 2001).

Our results provide a basis for explaining more complex processes which affect the health of those animals subjected to marine contaminants, which can provoke a loss of immunological resistance and play a role in the incidence of infectious diseases or the high prevalence of cancers.

Acknowledgements

This work was supported by the Canada Research Chair in Environmental Immunotoxicology (M.F.). The expert technical assistance of Marlene Fortier was greatly appreciated.

Table 3. Contaminant levels in blood of pinnipeds

Contaminant	Blood concentration	Species, sources
Zn	3.4 ± 0.5 mg/L	Harbour seal *Phoca vitulina*
	2.6-6.2 mg/L	Griesel et al. (2008)
	n = 85	Includes data from Grielel et al. (2006)
	3.13 ± 0.11 µg/g	Southern elephant seal *Mirounga leonina*
	n = 6	Baraj et al. (2001)
	3150 ± 0.04 µg/L	Grey seal *Halichoerus grypus*
	n = 3	Kakuschke et al. (2006)
Fe	733 ± 86 mg/L	Harbour seal *Phoca vitulina*
	485 – 1136 mg/L	Griesel et al. (2008)
	n = 85	Includes data from Grielel et al. (2006)
	700.6 ± 28.5 µg/g	Southern elephant seal *Mirounga leonina*
	n = 6	Baraj et al. (2001)
V	0.88 µg/L	Harbour seal *Phoca vitulina*
	<0.05 – 3.06 µg/L	Griesel et al. (2008)
	n = 28	
	0.014 µg/g	Northern fur seal *Callorhinus ursinus*
	n = 2	Saeki et al. (1999)
	1.05 ± 0.07 µg/L	Grey seal *Halichoerus grypus*
	n = 3	Kakuschke et al. (2006)
As	175 µg/L	Harbour seal *Phoca vitulina*
	42 – 592 µg/L	Griesel et al. (2008)
	n = 28	
	108 ± 0.03 µg/L	Grey seal *Halichoerus grypus*
	n = 3	Kakuschke et al. (2006)

Contaminant	Blood concentrations	Species, sources
Se	885 µg/L	Harbour seal *Phoca vitulina*
	518 – 2261 µg/L	Griesel et al. (2008)
	n = 85	
	1119 ± 0.02 µg/L	Grey seal *Halichoerus grypus*
	n = 3	Kakuschke et al. (2006)
	665 – 1449 µg/L	Pilot whale *Globicephala melas*
	n = 6	Nielsen et al. (2000)
	324 – 729 µg/L	Sperm whale *Physeter catodon*
	n = 4	Nielsen et al. (2000)
Cr	13.62 ± 1.28 µg/L	Grey seal *Halichoerus grypus*
	n = 3	Kakuschke et al. (2006)
	19.3 µg/L	Harbour seal *Phoca vitulina*
	7.42 ± 50.2 µg/L	Kakuschke et al. (2005)
	n = 13	
	7.10 ± 1.79 ng/g	Southern elephant seal *Mirounga leonina*
	n = 6	Baraj et al. (2001)
Ag	9.88 ± 0.25 µg/L	Grey seal *Halichoerus grypus*
	n = 3	Kakuschke et al. (2006)
	10.6 µg/L	Harbour seal *Phoca vitulina*
	<1.8 – 46.8 µg/L	Kakuschke et al. (2005)
	n = 13	
B[a]P	6.03 ± 4.86 ng/g	Southern sea lion *Otaria flavescens*
	n = 3	Marsili et al. (1997)
	3.75 ± 3.42 ng/g	Southern sea lion *Otaria flavescens*
	n = 3	Marsili et al. (1997)

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6. DISCUSSION GÉNÉRALE

L'utilisation croissante de substances polluantes par l'homme, leur présence dans l'environnement marin et leurs effets sur les organismes aquatiques constituent l'un des problèmes majeurs du XXIème siècle. Ce travail de thèse s'inscrit dans ce contexte. Les objectifs principaux de ce travail visaient 1) à approfondir notre connaissance de l'immunologie des pinnipèdes; 2) d'étudier la relation entre présence de polluants et immunomodulation; 3) d'évaluer la dynamique de transfert des contaminants entre une mère et son jeune durant la lactation; 4) de déterminer la distribution géographique des contaminants entre les espèces de pinnipèdes de l'Estuaire et du Golfe du Saint-Laurent; 5) d'établir si un modèle de transfert des contaminants à travers un réseau trophique de l'Estuaire du Saint-Laurent basé sur les isotopes stables était envisageable. Les modèles biologiques choisis pour répondre à ces objectifs étaient les phoques communs, gris et du Groenland. Pour le modèle de transfert des contaminants à travers le réseau trophique seul le béluga est utilisé dans un premier temps.

Des discussions détaillées concernant les comparaisons par rapport aux méthodes utilisées ou à la littérature se trouvent à la fin de chaque article, seul ici les liens entre les articles seront discutés.

Bien que le système immunitaire des mammifères marins soit de plus en plus étudié, certaines informations manquent encore. Ainsi le développement du système immunitaire chez les nouveau-nés et son évolution à travers le cycle de vie sont pour l'instant peu connus. De même, les réponses de différents organes du système immunitaire suite à une exposition aux contaminants n'ont jamais été évaluées chez les pinnipèdes.

Le système immunitaire a pour fonction de protéger l'hôte contre tout agent potentiellement pathogène. Nos travaux démontrent que des changements en lien avec l'âge se produisent dans le système immunitaire inné des pinnipèdes. Tandis que l'activité et l'efficacité phagocytaire augmente durant la lactation chez les phoques nouveau-nés, suggérant un système immunitaire compétent; chez les femelles adultes de phoque commun, l'activité phagocytaire diminue avec l'âge. Ces résultats suggèrent que dépendamment de l'âge, la capacité des organismes à répondre aux pathogènes varie. Ainsi, la résistance aux pathogènes diminuera donc avec l'âge puisque l'activité phagocytaire va se trouver diminuée, les cellules seront alors moins capables d'éliminer les pathogènes.

Les expériences réalisées *in vitro* (sur des lymphocytes T issus de différents organes immunitaires prélevés sur un jeune phoque gris femelle) ont permis de

démontrer que lors d'une exposition aux contaminants, la prolifération des lymphocytes T varie selon la provenance des cellules. Les réponses différent selon que lymphocytes T proviennent du thymus, d'un ganglion lymphatique ou du sang, permette de déterminer une action spécifique d'un polluant donné sur la prolifération des lymphocytes T d'un tissu cible. Ainsi, les lymphocytes T provenant du thymus semblent être plus sensibles aux BPC que ceux du sang ou du ganglion lymphatique.

Ces deux articles (4.1 et 4.2) démontrent que la réponse du système immunitaire varie selon l'âge de l'individu mais également selon l'organe analysé. Il serait intéressant d'effectuer une comparaison chez des nouveau-nés de plusieurs espèces afin de définir les variations inter-espèces de résistance de l'hôte aux différents pathogènes rencontrés dès la naissance.

Afin de mieux définir la transmission des contaminants entre une mère et son jeune durant la lactation, un modèle biologique a été choisi, le phoque du Groenland. Nos résultats démontrent que le transfert des pesticides, PBDE et BPC est régis par leur liposolubilité, et qu'un transfert sélectif se produit durant la production du lait conduisant à des patrons de contaminants différents entre la mère et son jeune. Ce transfert de POP démontre l'importance de la lactation comme une voie d'excrétion des contaminants pour les mères et en parallèle, une voie d'exposition à ces derniers chez leurs jeunes. Il ressort que les nouveau-nés sont exposés aux contaminants durant un stade critique de leur développement.

Les analyses de PBDE dans le gras et le sérum de trois espèces de phoques de l'Estuaire et du Golfe du Saint-Laurent ont permis de mettre en évidence que les nouveau-nés provenant de l'Estuaire sont cinq fois plus contaminés que ceux du Golfe dû à l'apport fluvial de PBDE. Cependant, dans le Golfe, les concentrations de PBDE sont similaires entre les nouveau-nés des trois espèces analysées (phoques commun, gris et du Groenland). De plus, le sérum ayant des concentrations de $\sum$PBDE similaires à celles du gras, cette matrice (issue d'une technique moins invasive qu'une biopsie de gras) pourrait être utilisée préférentiellement au gras dans les études de suivis environnementaux des nouveau-nés durant la lactation. Nous avons de plus démontré que la similarité dans les concentrations de PBDE chez les nouveau-nés des trois espèces de phoques du Golfe du Saint-Laurent pouvait être expliquée par la diète de leurs mères. Nos travaux démontrent que les pinnipèdes de l'Estuaire du Saint-Laurent sont susceptibles d'être exposés dès leur plus jeune âge à des niveaux toxiques de contaminants.

Bien que généralement l'exposition à un contaminant conduit à son accumulation dans l'organisme, il arrive que certains contaminants s'accumulent peu (l'organisme les excrètent de façon efficace) mais ils passent cependant à travers l'organisme et pourraient donc avoir des effets néfastes sur la santé de l'animal. De plus, certains stress physiologiques tels que la gestation, la lactation, la mue (associé à un jeûne) vont remettre en suspension des contaminants lipophiles entreposés dans les tissus graisseux de l'animal et les cellules vont donc être ré-exposés à ces contaminants. Il serait intéressant de faire un suivi de la charge en contaminants durant ces stress physiologiques et l'impact que pourraient avoir leurs concentrations sur le système immunitaire (il faudrait cependant différencier l'impact sur le système immunitaire que produit le stress physiologique).

Enfin, notre étude préliminaire démontre qu'il est envisageable d'utiliser les isotopes stables d'azote pour évaluer le transfert des butylétains à travers le réseau trophique de l'Estuaire du Saint-Laurent. La base de données sera complétée par la suite afin de mieux définir les différents réseaux trophiques présents dans l'Estuaire du St. Laurent. Utiliser les isotopes stables pour le transfert des butylétains à travers un réseau trophique apparaît prometteur. Cependant, il apparait important de compléter cette approche en suivant différents organes pour chaque espèce analysée, puisque les valeurs en isotopes stables vont différer entre les organes. Une suite possible à donner à ce travail serait également d'appliquer des facteurs de biomagnification (prédateur/proie) pour mieux définir l'évolution des butylétains à travers le réseau trophique.

Les expositions *in vitro* (sur des leucocytes prélevés directement de phoques communs) ont été largement utilisées afin d'évaluer chez le phoque commun le risque associé à l'exposition aux composés toxiques sur différents paramètres immunitaires sélectionnés. L'utilisation d'une lignée cellulaire (11B7501 issue d'un phoque commun) présente plusieurs avantages pour prévoir la toxicité. Tout d'abord elle provient d'un phoque commun et permet donc d'éviter les différences d'espèces, ensuite elle est issue directement des cellules mononucléaires du sang périphérique (prélevées sur un mâle ayant un lymphome/leucémie lymphoblastique). Néanmoins, il est vrai que certaines divergences existent entre les lignées cellulaires et les cellules normales, notamment le fait que les lignées cellulaires sont habituellement aneuploïdes (nombre de chromosomes varie d'une cellule à l'autre). Des tests sont donc encore nécessaires afin d'établir jusqu'à quel point la lignée cellulaire 11B7501 pourrait simuler les réponses de

cellules normales de phoque commun suite à une expositon aux contaminants. Ainsi, il est possible que les mécanismes de prolifération cellulaire soient différemment affectés, suite à une exposition à des toxiques, selon que les cellules soient normales ou issues du lymphome (où les processus sont déjà déréglés, permettant aux cellules de se diviser constamment). Les difficultés majeures liées à la toxicologie chez les mammifères marins sont les contraintes éthiques et logistiques rendant une étude sur le terrain excessivement coûteuse. S'il s'avère que cette lignée cellulaire (11B7501) simule de façon acceptable les réponses de cellules immunitaires normales alors ce problème pourrait être contourné.

Nous avons mis en évidence lors de travaux réalisées sur les butylétains que les phoques communs adultes et nouveau-nés différaient dans leurs réponses suite à une exposition *in vitro* (sur des leukocytes issus directement de phoques communs) au TBT ou DBT. Les fonctions immunitaires testées (phagocytose, prolifération lymphoblastique et activité cytotoxique des cellules NK) étaient plus touchées par l'exposition aux butylétains chez les adultes que chez les nouveau-nés. Bien que les nouveau-nés capturés lors de cette étude ne présentaient pas de niveaux immunotoxiques de butylétains, nous proposons que chez les adultes, les concentrations seraient suffisantes pour induire des effets immunotoxiques.

Les expériences menées avec les polybromés diphényles éthers (PBDE) ont permis de mettre en évidence leurs effets sur le système immunitaire du phoque commun. Les trois congénères testés (BDE-47, -99 et -153) affectent une fonction immunitaire innée importante des granulocytes, la flambée oxydative, qui génère des espèces oxygénées réactives (EOR) utilisées pour tuer les microorganismes ingérés. En présence de la plus forte concentration testée (12 µM) les niveaux d'EOR augmentent chez les adultes et les nouveau-nés. D'autre part, le niveau intracellulaire de thiols et l'activité et l'efficacité phagocytaire diminuent suite à la plus forte exposition chez les trois modèles testés, adultes, nouveau-nés et lignée cellulaire. Ces expériences ont également permis de démontrer que les effets observés sont similaires entre les phoques commun adultes et nouveau-nés et la lignée cellulaire 11B7501 issue d'un phoque commun. Cette dernière pouvant donc servir de modèle.

Basé sur cette constatation, avec la lignée cellulaire (11B7501) de nouveaux tests ont été développés, qui se sont avérés pertinents au cours des expériences réalisées *in vitro* (sur la lignée de cellules). Cette approche est originale, car les études similaires nécessitent l'utilisation de cellules immunitaires prélevées peu de temps

auparavant sur l'animal. Cependant, cette lignée cellulaire ne comportant que deux types cellulaires (lymphocytes B et B/macrophage), certaines fonctions du système immunitaire ne peuvent être étudiés. Il serait intéressant de mettre au point d'autres lignées cellulaires afin de disposer d'un nombre plus important de paramètres à tester, dans le but de pouvoir relier un polluant ou une catégorie de polluants à une réponse et des biomarqueurs spécifiques.

La présente étude a permis de démontrer qu'à des niveaux environnementaux (retrouvés dans le sang des pinnipèdes), le B[a]P, le sélénium, le vanadium et l'arsenic induisent aussi bien des effets immunotoxiques (diminution de la prolifération lymphoblastique et de la phagocytose) que génotoxiques (induction de micronuclei).

Les études *in vitro* présentent plusieurs avantages comparés aux études *in vivo*, notamment pour mesurer et caractériser, anticiper les effets et le devenir de molécules chimiques spécifiques chez les organismes. En effet, une étude *in vivo* doit prendre en compte tous les contaminants présents dans l'organisme et donc les effets d'une molécule ou d'un groupe de molécules sont plus difficilement décelables. Cependant, l'étude *in vitro* ne permet pas de savoir comment réagira réellement l'organisme quand il est réellement exposé au contaminant. Cependant, basés sur nos résultats, en supposant que suite à une contamination, les cellules se comportent *in vivo* comme elles se comportent *in vitro* (sur les leukocytes et la lignée cellulaire), il ressort que le système immunitaire du phoque commun peut-être affaibli suite à l'exposition permanente aux contaminants retrouvés dans son environnement. Le système immunitaire pourrait alors être moins performant face à une exposition à des pathogènes environnementaux (comme il a été suggéré pour les épizooties à morbillivirus). Ainsi, dans le contexte de mémoire immunologique, la capacité de prolifération des lymphocytes présentant l'antigène d'un pathogène préalablement rencontré, pourrait se trouver diminuer suite à l'exposition à des contaminants et le système immunitaire pourrait donc être moins en mesure de se défendre. Suite à une approche *in vitro*, des études *in vivo* sont aussi à envisager afin de mieux définir les réponses du système immunitaire. Une solution serait de capturer des phoques de différentes classes d'âge et d'analyser pour chaque classe d'âge plusieurs paramètres immunitaires en lien avec les concentrations de PBDE, BPC, métaux lourds et BTs retrouvés dans le sang. Cette approche permettrait de mieux estimer l'association entre un contaminant et son effet sur un paramètre immunitaire donné. D'autres xénobiotiques tels que les substances médicamenteuses pourraient être testées. En effet, des substances de ce type sont rejetées en quantités

importantes dans l'environnement (Halling-Sorensen et al., 1998) et, à l'heure actuelle, très peu d'études ont tenté de définir leur impact sur les organismes (Halling-Sorensen et al., 1998).

L'ensemble de ce travail a apporté des informations concernant le système immunitaire des pinnipèdes, ses réponses suite à une exposition à des contaminants, ainsi que le transfert de ces derniers entre la mère et son jeune. Il s'avère que les phoques vivant dans l'Estuaire du St-Laurent sont plus exposées aux PBDEs que les phoques du Golfe du St-Laurent. Cette distinction géographique pourrait impliquer des effets néfastes sur le système immunitaire des adultes de l'Estuaire du St-Laurent (les valeurs retrouvées chez les nouveaux-nés sont cependant trop faibles pour induire des effets). *In vitro*, il a été démontré que les concentrations induisant un effet sur le sytème immunitaire sont similaires entre les adultes et les nouveau-nés. De plus, la contamination en BT est faible chez les nouveau-nés du Parc du Bic mais pourrait s'avérait élever chez les colonies résident à l'entrée du Saguenay (connu pour être contaminé en BT comme démontré par les concentrations élevées reportées chez les bélugas) et une diminution de leurs performances immunitaires pourraient donc être observées. De plus, *in vitro*, il a été démontré qu'à concentrations égales, le système immunitaire des nouveau-nés semble plus résistant aux BT.

À plus long terme, une perspective de ce travail de thèse est de pouvoir utiliser les concentrations mesurées chez les nouveau-nés ou les adultes et de les associer à des effets potentiels sur le système immunitaire en formation ou déjà formé. En combinant cette approche avec une meilleure connaissance des relations entre les différents types de polluants et le développement possible de différentes maladies, nous pourrons ainsi disposer d'outils permettant de prédire l'évolution d'une épizootie (apparition d'une maladie, son développement et son extension).

Ce travail a donc permis de définir le lien entre toxicologie et immunologie. Il ressort de notre étude que ce lien est fondamental et qu'une meilleure connaissance des effets des polluants sur le système immunitaire et sa capacité à répondre aux maladies infectieuses, pourrait permettre d'empêcher, ou du moins de mieux comprendre, l'apparition de nouvelles épizooties.

Référence

Halling-Sorensen B., Nielsen S.N., Lanzky P.F., Ingerslev F., Lützhoft H.C.H., Jorgensen S.E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment-a review. Chemosphere 26, 357-393.

7. LA CONTRIBUTION A L'AVANCEMENT DES CONNAISSANCES

Les trois thèmes majeurs abordés dans cette étude sont l'immunologie, la dynamique des contaminants et l'immunotoxicologie.

Mes travaux ont permis de mieux comprendre l'immunologie chez les différentes espèces de phoques tels que les phoques communs (*Phoca vitulina*), gris (*Halichoerus grypus*) et du Groenland (*Phoca groenlandica*). Dans cette thèse nous avons déterminé que des changements en lien avec l'âge se produisaient dans le système immunitaire inné des pinnipèdes, suggèrant que dépendamment de l'âge, la capacité des organismes à répondre aux pathogènes varie. De plus, des expériences réalisées *in vitro* (sur des lymphocytes T issus de différents organes immunitaires directement prélevés sur un jeune phoque gris femelle) ont permis de démontrer que, pour cet individu, les réponses diffèraient selon que les lymphocytes T provenaient du thymus, d'un ganglion lymphatique ou du sang. Il serait intéressant de refaire l'étude avec un n plus important afin de définir si les réponses observées étaient dû à l'individu ou sont représentative de l'espèce, permettant ainsi d'envisager de déterminer une action spécifique d'un polluant donné sur la prolifération des lymphocytes T d'un tissu cible.

Nos analyses de PBDE dans le gras et le sérum des trois espèces de phoques de l'Estuaire et du Golfe du Saint-Laurent ont mis en évidence que les nouveau-nés provenant de l'Estuaire sont cinq fois plus contaminés que ceux du Golfe. De plus aussi bien le sérum que le gras peuvent être utilisés pour le suivi de la contamination aux PBDE chez les nouveau-nés. Afin de mieux définir la transmission des contaminants entre une mère et son jeune durant la lactation, un modèle biologique a été choisi, le phoque du Groenland. Nos résultats démontrent que le transfert des pesticides, PBDE et BPC sont régis par leur liposolubilité, et qu'un transfert sélectif se produit durant la production du lait conduisant à des patrons de contaminants différents entre la mère et son jeune.

Finalement, nous avons établi qu'il est envisageable d'utiliser les isotopes stables d'azote pour évaluer le transfert des butylétains à travers le réseau trophique de l'Estuaire du Saint-Laurent et nous avons démontrer une bioamplification de ces composés à travers le réseau trophique étudié.

Nous avons mis en évidence lors de travaux *in vitro* que les fonctions immunitaires sont affectées suite à une exposition à des contaminants. Cependant, les réponses divergent entre les adultes et les nouveau-nés chez le phoque commun. Nous avons déterminés que les contaminants présentant un aspect toxique sont le TBT, le

DBT, certains congénères de PBDE, les BPC et le B[a]P ainsi que certains métaux lourds tels que le sélénium, le vanadium et l'arsenic.

Dans l'ensemble, nos travaux suggérent que le système immunitaire du phoque commun peut-être affaibli suite à l'exposition permanente aux contaminants retrouvés dans son environnement. De plus, il ressort que les nouveau-nés sont exposés aux contaminants durant un stade critique de leur développement.

8. ANNEXES

(Articles écrits durant la thèse et lettres des éditeurs pour les articles soumis et acceptés)

ANNEXE 1 : Comparative sensitivity of harbour and grey seals to several environmental contaminants using *in vitro* exposure

Article 9 : *Dufresne M. M., Frouin H., Pillet S., Lesage V., De Guise S., Fournier M. Comparative sensitivy of harbour and grey seals to several environmental contaminants using in vitro exposure. Marine Pollution Bulletin. (Article accepté le 22 octobre 2009).*
Mon rôle a consisté à rédiger l'article à titre de second auteur en me basant sur les résultats préalablement obtenus et traités par M. Dufresne.

RÉSUMÉ

Dans cette étude, nous étudions les effets du chlorure de cadmium ($CdCl_2$), du chlorure de mercure ($HgCl_2$), du chlorure de methylmercure (CH_3HgCl) et des PCB sur la prolifération lymphoblastique chez les phocidés. Les lymphocytes isolés de phoques gris et commun ont été exposés in vitro à différentes concentrations de contaminants. Une réduction de la viabilité se produit quand les cellules sont exposées à 10^{-4} M de $HgCl_2$ ou de CH_3HgCl ou à 50 ppm d'Aroclor 1254. Aussi bien chez le phoque gris que chez le phoque commun, la prolifération des lymphocytes T est supprimée quand les cellules sont incubées avec 5 x 10^{-5} M de $CdCl_2$ ou 10^{-4} M de $HgCl_2$. La prolifération est inhibée avec le CH_3HgCl dès 10^{-6} M chez les phoques gris et dès 10^{-5} M chez les phoques communs. L'Aroclor 1254 réduit la prolifération lymphocytaire à 15 ppm chez les phoques gris. Aussi bien pour le phoque commun que le phoque gris, le CH_3HgCl était 10 fois plus immunotoxique que le $HgCl_2$. Avec l'IC_{50}, les composés chimiques peuvent être classés en terme de toxicité dans l'ordre suivant : CH_3HgCl >$CdCl_2$ >$HgCl_2$ >Aroclor 1254.

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Marine Pollution Bulletin xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Comparative sensitivity of harbour and grey seals to several environmental contaminants using *in vitro* exposure

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ARTICLE INFO

Keywords:
Immunotoxicity
Mercury
Cadmium
PCBs
Harbour seals
Grey seals

ABSTRACT

In this study, we investigated the effects of cadmium chloride ($CdCl_2$), mercury chloride ($HgCl_2$), methylmercury chloride (CH_3HgCl), and PCBs on lymphocyte proliferation in phocids. PBMCs isolated from harbour and grey seals were exposed *in vitro* to varying concentrations of contaminants. A reduction of viability occurred when cells were exposed to 10^{-4} M $HgCl_2$ or CH_3HgCl or to 50 ppm of Aroclor 1254. In both grey and harbour seals, T-lymphocyte proliferation was suppressed when their cells were incubated with 5×10^{-5} M $CdCl_2$ or 10^{-4} M $HgCl_2$. An inhibition of proliferation occurred with CH_3HgCl from 10^{-6} M in grey seals and from 10^{-5} M in harbour seals. In grey seals, Aroclor 1254 reduced lymphocyte proliferation at 15 ppm. In both harbour and grey seals, CH_3HgCl was ten times more immunotoxic that $HgCl_2$. From IC_{50}, chemicals were ranked in terms of toxicity as followed: $CH_3HgCl > CdCl_2 > HgCl_2 >$ Aroclor 1254.

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1. Introduction

In the St. Lawrence Estuary, harbour seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) overlap in their distribution. The harbour seal is a permanent resident of the St. Lawrence Estuary (Lesage et al., 2004). Grey seals are also found at coastal haulout sites, but normally are only found in the area during summer, leaving the St. Lawrence Estuary in November to overwinter in the Gulf of St. Lawrence or on the Atlantic coast of Canada (Lavigeur and Hammill, 1993).

Knowledge about pinniped immunology advanced rapidly following the 1988 phocine distemper virus (PDV) epizootic in Northern Europe (Dietz et al., 1989). A previous study suggested that grey and harbour seals differ in their susceptibility to certain diseases. For example, harbour and grey seals have become infected with influenza virus and morbillivirus (PDV), and both seroconverted following these infections (Geraci et al., 1982; Kennedy et al., 1989; Duignan et al., 1995). However, harbour seals often became fatally ill, while grey seals were much less likely to do so (Geraci et al., 1984; Harder et al., 1990; Heide-Jørgensen et al., 1992). The difference in susceptibility of the two seal species to viral infection may indicate a difference in immune response to viral epitopes or a different tropism of the virus. Duignan et al. (1997) demonstrated that the antibody response of grey seals appeared to be more competent than that of harbour seals with respect to morbillivirus antigens.

Mass mortalities among seals inhabiting contaminated marine areas have led to speculation about the possible involvement of immunosuppression (Van Loveren et al., 2000) associated with the finding of relatively large contaminant burdens in these animals (Hall et al., 1992). An imbalance of the immune system caused by pollutants has been suggested to play a role in the development of infectious diseases (Bennett et al., 2001; Jepson et al., 2005). To characterize cellular immunity of marine mammals and/or the effects of pollutants, assays have been developed and applied to various parameters, including natural killer cell activity, phagocytosis, cytokine expression, and lymphocyte proliferation (De Guise et al., 1995; Pillet et al., 2000; Lalancette et al., 2003; Hammond et al., 2005; Camara Pellisso et al., 2008; Fonfara et al., 2008; Frouin et al., 2008).

An increasing "weight of evidence" suggested that environmental contaminants and PCBs in particular, contributed to the severity of the 1988 event (Hall et al., 1992; Ross et al., 1996). However, possible immunosuppressive actions of heavy metals cannot be ruled out (Das et al., 2008; Kakuschke et al., 2008). Indeed, concentrations of mercury and cadmium in marine mammals have increased considerably since the turn of the twentieth century (Wagemann et al., 1990). Few cadmium toxic effects have yet been reported in marine mammals (De Guise et al., 1996) despite their high levels encountered in several species, suggesting highly efficient detoxification mechanisms (Dietz et al., 1998). While

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0025-326X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marpolbul.2009.10.019

Please cite this article in press as: Dufresne, M.M., et al. Comparative sensitivity of harbour and grey seals to several environmental contaminants using *in vitro* exposure. Mar. Pollut. Bull. (2009), doi:10.1016/j.marpolbul.2009.10.019

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inorganic mercury (Hg) is the major source of Hg to most aquatic systems, it is methylmercury (CH_3Hg) which bioaccumulates and biomagnifies at all trophic levels in marine food webs (Gray, 2002) and which is the source of severe toxicological effects on wildlife (reviewed by Wolfe et al., 1998). Exposure to mercury impairs specific immune defenses of many species including marine mammals (De Guise et al., 1996; Pillet et al., 2000; Lalancette et al., 2003; Camara Pellisso et al., 2008; Das et al., 2008).

The purpose of this study was to investigate the immunotoxicity of multiple chemicals to which is commonly exposed marine mammal immune system. Mercury chloride ($HgCl_2$), methylmercury chloride (CH_3HgCl), cadmium chloride ($CdCl_2$), and the PCB congener Aroclor 1254 were selected because they are common anthropogenic contaminants in aquatic ecosystems, notably in the St. Lawrence Estuary (Lee et al., 1999).

2. Materials and methods

2.1. Animal capture, handling, and sample collection

Blood samples were collected in 1996 from three adult female grey seals, held at the Zoological Society of Granby (Granby, Qc, Canada) and from 4 harbour seals live-captured in 1996 in the St. Lawrence Estuary, Quebec, Canada (between 47°56′ N and 48°41′ N) (details in Table 1). Harbour seals included two subadult (one year old) male, one subadult (one year old) female, and one adult (seven years old) female.

Wild animals were caught using a gill net and handled on a boat for blood sampling and identification, then released. Blood samples were drawn from the epidural vein into heparinized tubes (Vacutainer ©, Becton Dickinson) and was kept at 4 °C until the time of analyses, which occurred 6 h or less after sampling.

2.2. Chemicals

Stock solutions of $HgCl_2$ and $CdCl_2$ (Aldrich, St. Louis, MO) were prepared in distilled water at a concentration of 10^{-2} M. CH_3HgCl was obtained from Ultra Scientific (North Kingstown, RI, USA) and was first dissolved in alcohol and diluted in RPMI 1640 media (Gibco BRL, Mississauga, ON) to achieve a stock solution of 10^{-2} M. The Aroclor 1254 (Ultra Scientific, North Kingston, Ontario) was dissolved in RPMI supplemented with dimethyl sulfoxide (10% DMSO) to reach a concentration of 500 ppm. Subsequent dilutions were performed with RPMI 1640.

2.3. Isolation of mononuclear cells

Within 6 h after collection, blood was diluted in an equal volume of RPMI supplemented with 10% of heat inactivated fetal calf serum (Gibco BRL), 100 U/ml penicillin (Gibco BRL) and 100 μg/ml streptomycin (Gibco BRL) (complete RPMI). The peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Ficoll Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) at 900 g for 35 min at room temperature. The cells were then washed thrice and suspended in complete RPMI. Cells were counted and their viability assessed using acridine orange-propidium iodide with a fluorescence microscope.

2.4. Cytotoxicity

Viability assays were conducted only on grey seal PBMCs due to the limited sample volumes of harbour seals. Cells were adjusted to 5×10^6 cells/ml and deposited in a 96-well tissue culture plate. After 3 h the sample was transferred into a tube containing an equal volume of an isotonic solution (Hematall; Fisher Scientific, Montréal, Canada) and 50 μg/ml of propidium iodide. The fluorescence of approximately 10,000 cells was read by flow cytometry (FACScan; Becton Dickinson, San José, CA) and the data were analyzed by the LYSIS-II software (FACScan, Becton Dickinson). Results were expressed as the percentage of viable cells.

2.5. Proliferation

Lymphocyte proliferation was measured by 3^H-thymidine uptake. Briefly, PBMCs adjusted to a concentration of 5×10^6 cells/ml (80 μl) were plated with 80 μl of mitogen, to which was added either 20 μl of complete RPMI (control) or 20 μl of the contaminant to be tested (final concentration ranging from 10^{-9} M to 10^{-3} M for heavy metals and from 5 to 50 ppm for Aroclor 1254). For $CdCl_2$ and CH_3HgCl some of the concentrations were tested only on grey seal PBMCs due to the limited sample volumes of harbour seals. Similarly, only grey seals PBMCs were exposed to 5 to 50 ppm of Aroclor 1254. Cells were incubated 54 h at 37 °C and 5% CO_2 in 96-well tissue culture plates. The optimal concentration of mitogen used to stimulate T lymphocytes was 5 μg/ml concanavalin A (con A). Samples were tested in triplicate. After the 54 h incubation, 0.5 μCi (20 μl) of H^3-thymidine (6.7 Ci/mmol; ICN, Mississauga, Canada) were added in each well and plates were incubated for an additional 18 h. The DNA was collected on fiberglass filter with a Titerteck Cell Harvester and the amount of radioactivity incorporated was evaluated with a Beckman β-scintillation counter. The raw data were expressed in disintegration per minute (DPM), triplicates were averaged, and results were presented as percentage of the control.

2.6. Statistics

Differences between controls and treated groups were evaluated by one-way ANOVA followed by Dunnett's two-sided post hoc test. Student's t-tests were performed to identify potential differences between species for inhibition of 50% of the proliferation response (IC_{50}). Statistical analyses were performed using STATISTICA for windows (version 7.0, StatSoft Inc., 1995).

3. Results

3.1. Viability

Cytotoxic effect of cadmium, mercury, methylmercury and Aroclor 1254 was assessed using grey seal PBMCs. Cadmium

Table 1
Details of harbour seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) sampled in 1996 in this study.

Species	Location	Sex	Weight (kg)	Length (cm)	Age	Blood collection
Grey seal	Zoo (Granby)	F	165	No data	7	Superficial plantar flipper veins
Grey seal	Zoo (Granby)	F	180	No data	10	Superficial plantar flipper veins
Grey seal	Zoo (Granby)	F	115	No data	6	Superficial plantar flipper veins
Harbour seal	St. Lawrence Estuary	M	36	113	1	Epidural vein
Harbour seal	St. Lawrence Estuary	M	39	115	1	Epidural vein
Harbour seal	St. Lawrence Estuary	F	57	125.5	7	Epidural vein
Harbour seal	St. Lawrence Estuary	F	29	102	1	Epidural vein

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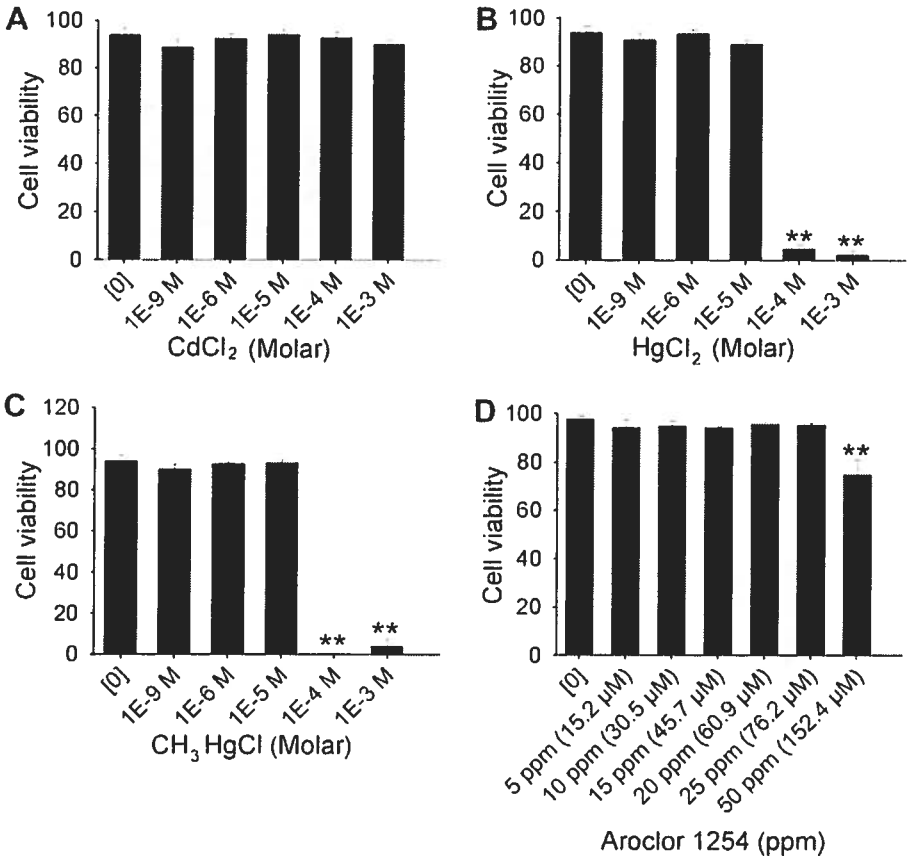

Fig. 1. Relative viability of captive grey seal peripheral blood mononuclear cells exposed 3 h *in vitro* to cadmium chloride (A), mercury chloride (B), methylmercury chloride (C) or Aroclor 1254 (D). Results are expressed as mean of live cells (± SEM). One-way ANOVA followed by Dunnett's two-sided post hoc test was performed to indicate statistical significant differences between exposed and control cells (**$p < 0.01$).

chloride (Fig. 1A) was not cytotoxic at the concentrations tested. However, $HgCl_2$ (Fig. 1B) significantly reduced cell viability ($p < 0.001$) by up to 99% at the highest concentrations (10^{-4} and 10^{-3} M). Methylmercury chloride (Fig. 1C) also significantly reduced cell viability ($p < 0.001$) by 95% at 10^{-4} and 10^{-3} M. Aroclor 1254 (Fig. 1D) significantly reduced cell viability ($p = 0.002$) by up to 25% only at the highest concentration (50 ppm or 152.4 µM).

3.2. Lymphocyte proliferation

The effects of exposure to heavy metals and PCBs on the proliferation of grey and harbour seal T lymphocytes are shown in Fig. 2. In both grey and harbour seals, the proliferative response of T lymphocytes exposed to $CdCl_2$was significantly inhibited ($p < 0.001$) at concentrations of 5×10^{-5} to 10^{-3} M. However, a concentration of 5×10^{-10} M $CdCl_2$ induced a significant 50% increase ($p = 0.014$) of the proliferative response of harbour seal T cells. Mercury chloride significantly inhibited the proliferative responses in both harbour ($p = 0.001$) and grey ($p < 0.001$) seal lymphocytes following *in vitro* exposures to concentrations of 10^{-4} M up to 10^{-3} M. A significant inhibition of T cell proliferation with CH_3HgCl occurred when exposed to 10^{-6} M up to 10^{-3} M in grey seals ($p < 0.001$) and to concentrations of 10^{-5} M up to 10^{-3} M in harbour seals ($p < 0.001$). The magnitude of heavy metal-induced decreases in T cell proliferation was consistently of 70% or greater.

For Aroclor 1254 a dose–response inhibition of T cell proliferation ($p < 0.001$) was observed and was statistically significant at concentrations varying from 15 ppm (45.7 µM) to 50 ppm (152.4 µM).

3.3. IC_{50}

Dose values for IC_{50} (Table 2) indicated a difference in proliferative responses between grey and harbour seals ($p = 0.001$) when exposed to CH_3HgCl, with grey seals being more sensitive than harbour seals. From these results, chemicals can be ranked in terms of toxicity as followed: $CH_3HgCl > CdCl_2 > HgCl_2 >$ Aroclor 1254.

4. Discussion

Results presented in this study clearly demonstrated that Hg, CH_3Hg, Cd, and PCBs can inhibit lymphoblastic proliferation in seals.

As a first step, the effects of $HgCl_2$ and $CdCl_2$ were examined, followed by the effects of CH_3HgCl. Then, the immunotoxicity of $HgCl_2$ and CH_3HgCl were compared. Finally, the effects of PCBs were examined. To conclude our study, blood concentrations of these contaminants reported in pinnipeds were analyzed considering their potential immunological impact.

In our experiment, the exposure to 10^{-4} M $HgCl_2$ and to 5×10^{-5} M $CdCl_2$ decreased lymphocyte proliferation in both harbour and grey seals. The inhibition of the proliferative response could be related to a higher cytotoxicity as observed after 3 h for $HgCl_2$ which was not the case for $CdCl_2$. Previous studies demonstrated that $HgCl_2$ decreased viability of bottlenose dolphin (*Tursiops truncatus*) lymphocytes at 10 ppm (3.7×10^{-5} M) (Camara Pellisso et al., 2008) and reduced viability of beluga (*Delphinapterus leucas*) thymocytes at 10^{-5} M (De Guise et al., 1996), a concentration which is one order of magnitude lower than in our study.

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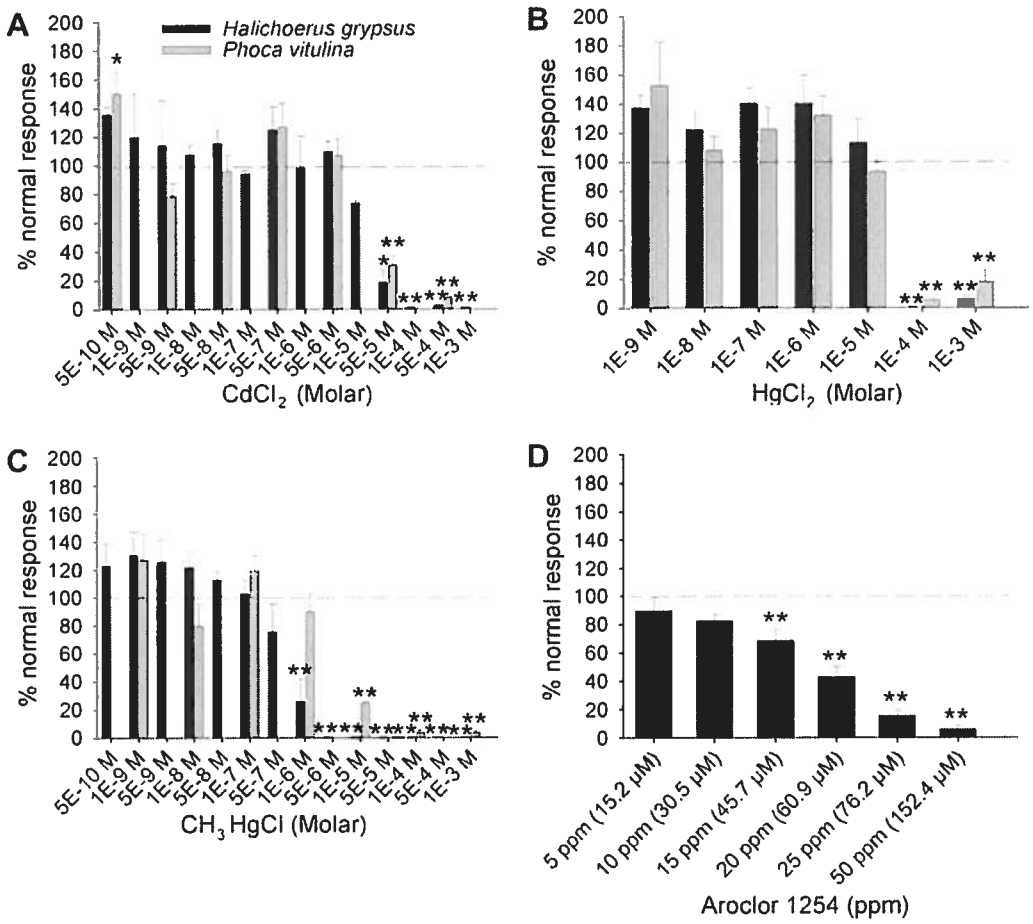

Fig. 2. Stimulation index of grey and harbour seal lymphocytes stimulated with con A and cultured in presence of cadmium chloride (A), mercury chloride (B), methylmercury chloride (C) or Aroclor 1254 (D). One-way ANOVA followed by Dunnett's two-sided post hoc tests were performed to indicate statistical significant differences between exposed and control cells (*$p < 0.05$, **$p < 0.01$).

Table 2
Immunosuppression dosage at 50% proliferation response of pollutants in harbour and grey seals.

Compound	Harbour seals	Grey seals
$CdCl_2$	$3.91 \times 10^{-5} \pm 0.58 \times 10^{-5}$ M (4)	$3.17 \times 10^{-5} \pm 0.31 \times 10^{-5}$ M (4)
CH_3HgCl	$6.84 \times 10^{-6} \pm 0.85 \times 10^{-6}$ M (3)*	$1.30 \times 10^{-6} \pm 1.11 \times 10^{-6}$ M (4)*
$HgCl_2$	$6.02 \times 10^{-5} \pm 0.37 \times 10^{-5}$ M (3)	$5.75 \times 10^{-5} \pm 1.37 \times 10^{-5}$ M (4)
Aroclor 1254		17.9 ppm (54.6 µM) ± 2.9 ppm (8.8 µM) (5)

Number of samples is given in brackets. *Significantly different $p < 0.05$.

Moreover, $HgCl_2$ inhibited proliferation of beluga whale thymocytes (De Guise et al., 1996) and of bottlenose dolphin lymphocytes (Camara Pellisso et al., 2008) at concentrations of 10^{-5} M and 1 ppm (3.7×10^{-6} M), respectively. Our results suggest that cells from seals are less sensitive to the effects of $HgCl_2$ than cells from cetaceans. In the present work, the inhibition of seal lymphocyte proliferation observed with 5×10^{-5} M of $CdCl_2$ is consistent with a previous investigation, which indicated a decrease in splenocyte proliferation in beluga whales at concentrations of 10^{-5} M of $CdCl_2$ (De Guise et al., 1996). With $CdCl_2$, an increase of lymphocyte proliferation was also observed at low doses (5×10^{-10} M) in harbour seals. Our results are consistent with a published study on an *in vitro* activation of sheep lymphocytes by low concentration (10^{-8} M) of $CdCl_2$ (Stec, 2001). These results show that cadmium enhanced lymphocyte proliferation at low levels of exposure, whereas higher one resulted in inhibition of DNA synthesis.

The proliferation of lymphocytes was significantly reduced by CH_3HgCl at 10^{-6} M in grey seals and at 10^{-5} M in harbour seals. The inhibition of the proliferative response could be linked to the cytotoxicity of CH_3HgCl for the highest concentrations (10^{-4} and 10^{-3} M) but not for 10^{-5} M and 10^{-6} M. A concentration of 10^{-5} M of CH_3HgCl was previously shown to decrease lymphoblastic proliferation in developing grey seals (Lalancette et al., 2003), which is one order of magnitude higher than in the present study. Moreover, Das et al. (2008) found that 10^{-6} M of CH_3HgCl was the critical concentration above which lymphocyte proliferation was compromised in harbour seals. This concentration is one order of magnitude lower than in our study.

Kakuschke et al. (2005, 2008) have previously demonstrated that an activation of lymphocyte proliferation induced by metals occurred in harbour seals. However, in Kakuschke et al. (2005) MeHg, HgCl, and Cd did not activate the lymphocyte proliferation in harbour seals and in Kakuschke et al. (2008) only MeHg and HgCl activated lymphocyte proliferation in some harbour seals but not all. In our study, lymphocyte proliferation of harbour and grey seals were not activated after exposure to $CdCl_2$, $HgCl_2$ or

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Table 3
Metal and organochlorine levels in blood of marine mammals.

Metal/ Organochlorine	Concentration	Localisation	Species, sources
Cd	<0.12–3.10 μg L^{-1} $n = 28$	Wadden Sea	Harbour seal *Phoca vitulina* Griesel et al. (2008)
	0.28 ± 0.01 μg L^{-1} $n = 1$	Wadden Sea	Grey seal *Halichoerus grypus* Kakuschke et al. (2006)
	3.8 ng g^{-1} $n = 6$	Antarctica	Southern elephant seal *Mirounga leonina* Baraj et al. (2001)
	<0.005–0.01 μg L^{-1} $n = 3$	Antarctica	Weddell seal *Leptonychotes weddellii* Yamamoto et al. (1987)
Hg	172 ± 143 μg L^{-1} $n = 22$	Wadden Sea	Harbour seal *Phoca vitulina* Das et al. (2008)
PCBs	201–1447 ng g^{-1} lipid $n = 7$	Greenland Sea	Harp seal *Phoca groenlandica* Lydersen et al. (2002)
	14.5 ± 2.6 ng g^{-1} $n = 19$	Alaska	Dam northern fur seal *Callorhinus ursinus* Beckmen et al. (1999)
	20.6 ± 6.9 ng g^{-1} $n = 48$	Alaska	Pup northern fur seal *Callorhinus ursinus* Beckmen et al. (1999)
	50 ± 10 ng g^{-1} $n = 14$	San Francisco Bay	Harbour seal *Phoca vitulina* Young et al. (1998)
	9.18–194 μg L^{-1} $n = 47$	North Sea	Harbour seal *Phoca vitulina* Weijs et al. (2009)

CH_3HgCl, suggesting that seals from the present study did not develop metal-specific hypersensitivity.

In both harbour and grey seals, the organic form of mercury (CH_3HgCl) was ten times more immunotoxic than the inorganic form ($HgCl_2$) (Table 2). Previous studies in beluga whale (De Guise et al., 1996) and in mice (Nakatsuru et al., 1985) have demonstrated that the proliferation of immune cells exposed to CH_3HgCl was affected at concentrations 10-fold lower than that of cells exposed to $HgCl_2$. In T lymphocytes, calcium (Ca^{2+}) mediates the effects of mitogens, such as con A or phytohaemagglutinin (PHA) (Freedman et al., 1975; Hesketh et al., 1983). However, in these cells, both organic and inorganic mercury can increase cytosolic levels of free calcium (Tan et al., 1993). Moreover, the effect of CH_3HgCl is rapid (one minute) and the increase of Ca^{2+} level is sustained in time, while $HgCl_2$ causes a slow rise (starts after several minutes) in intracellular $[Ca^{2+}]$. Thus, the differences between mercury compounds could be explained by different mechanisms of toxicity. Indeed, while $HgCl_2$-induced intracellular Ca^{2+} overload is due, primarily, to enhanced Ca^{2+} entry through Ca^{2+} channels, CH_3HgCl causes both calcium entry into the cell and calcium mobilization from intracellular stores (Tan et al., 1993).

Harbour seal cells appear less sensitive to methyl mercury at concentrations that have a detrimental effect on lymphocyte proliferation in grey seals. This observation is in contrast to a previous study. Hammond et al. (2005) investigated the effects of PCBs on innate immune functions in phocid seals and observed that exposure to PCBs significantly reduced immune functions of harbour seal polymorphonuclear cells *in vitro* (phagocytosis and respiratory burst activity), but had no effect on the same cells in grey seals from sympatric wild populations. Our results could suggest that in grey seals adaptative immune functions are more sensitive to contaminants than in harbour seals. It is also possible that grey and harbour seals exhibit differential sensitivity to immunotoxicity of different types of contaminants. In the present work, harbour seals were wild animals sampled in the St. Lawrence Estuary whereas grey seals were captive animals. It is possible that lymphocytes from wild seals environmentally exposed to the organic form of mercury could be less sensitive than lymphocytes from captive seals with no chronic exposure to this environmental pollutant.

In a third time, the effects of PCBs were studied. Aroclor 1254 inhibited the proliferative responses in grey seal PBMCs following *in vitro* exposures at concentrations of 15–50 ppm (45.7–152.4 μM). Aroclor 1254 is a commercial mixture in which the most abundant homolog groups are the tetra to heptachlorinated biphenyls (Frame et al., 1996). Data from a previous study indicated that con A-induced lymphocyte proliferation in bottlenose dolphins was negatively correlated with whole blood concentrations of tetrachlorinated to octachlorinated biphenyls (Lahvis et al., 1995). A study of exposure to PCBs and its toxic effects to pup harbour seals from British Columbia, Canada, showed that the proliferative responses of T lymphocytes, but not of B lymphocytes were significantly reduced in more contaminated animals (Mos et al., 2006).

Blood concentrations of cadmium and mercury measured in pinnipeds (Table 3) were much lower than those affecting lymphocyte proliferation in the present study. Knowing that in the blood of marine mammals Hg is mainly under a methylated form (up to 90%) (Woshner et al., 2008) and that mean of total Hg concentration in blood of harbour seals was 172 μg/L (around 10^{-6} M), methylmercury levels could be sufficient to affect lymphocyte proliferation, as was the case upon *in vitro* exposure in the present study. Finally, Aroclor 1254 induced an inhibition of lymphoblastic proliferation at concentrations (15–50 ppm) superior to these reported in the blood of pinnipeds (Table 3).

5. Conclusion

We have demonstrated that $CdCl_2$, $HgCl_2$ as well as CH_3HgCl and Aroclor 1254 can be immunotoxic for seal lymphocytes. Moreover, our results indicate that lymphocytes are differently affected by exposure to CH_3HgCl with respect to seal species. These findings could have important outcomes for using proliferative assay as a biomarker of environmental exposure as well as in understanding the mechanisms that could lead to the modulation of the responses resulting from the long-term exposure to xenobiotics in the environment.

Acknowledgements

The authors want to express their gratitude to the Zoological Society of Granby and especially to Dr. Clement Lanthier for providing grey seal blood samples. This work was supported by Fisheries and Oceans Canada through the St. Lawrence Vision 2000 fund and the US Environmental Protection Agency. MD was a

Please cite this article in press as: Dufresne, M.M., et al. Comparative sensitivity of harbour and grey seals to several environmental contaminants using *in vitro* exposure. Mar. Pollut. Bull. (2009), doi:10.1016/j.marpolbul.2009.10.019

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M.Sc. student supported by NSERC Canada whereas SP was supported by a grant from the Fonds pour la Recherche dans l'Industrie et l'Agriculture (Belgium). The authors wish to acknowledge Dr. Mike Hammill for field assistance and for providing comments on this manuscript.

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Please cite this article in press as: Dufresne, M.M., et al. Comparative sensitivity of harbour and grey seals to several environmental contaminants using *in vitro* exposure. Mar. Pollut. Bull. (2009), doi:10.1016/j.marpolbul.2009.10.019

ANNEXE 2 : Toxicological effects of mouse diet contaminated with tributyltin on the immune and neurological systems of C57Bl/6 mice

Article 10 : *M. Fortier, H. Frouin, A. Cloutier, M. Arseneault, C. Ramassamy, B. Badiwa-Bizowe, R. St.-Louis, E. Pelletier, M. Fournier. Toxicological effects of mouse diet contaminated with tributyltin on the immune and neurological systems of C57Bl/6 mice. Toxicological and Environmental Chemistry. (Accepté le 2 juin 2009).*

Mon rôle a consisté à participer à la rédaction de cet article.

RÉSUMÉ

Les composés organoétains, utilisés notamment comme agent antisalissures, sont connus pour se bioaccumuler dans la chaîne alimentaire. Parmi les composés organoétains, le tributyltin (TBT), un contaminant toxique et largement répandu, est devenu une menace sérieuse dans la pollution environnementale et est suspecté d'être immunotoxique chez les animaux. Le but de cette étude était d'estimer les effets de 80µg de TBT par kilogramme de poids corporelle, introduit dans la nourriture, sur les systèmes immunitaire et neurologiques de la souris C57Bl/6. Les données montrent que le TBT augmente la prolifération des lymphocytes T et B aussi bien chez les femelles adultes et juvéniles que les mâles juvéniles, mais il diminue la prolifération des lymphocytes T et B chez les mâles adultes. L'activité des macrophages chez les juvéniles (mâles et femelles) est plus élevée que chez les adultes dès deux sexes. L'activité cytotoxique des cellules NK est aussi augmentée chez les animaux de tous âges comparés au groupe témoin. Dans le cerveau, nous observons la présence de TBT dans l'hippocampe, le striatum, le cortex et le cervelet aussi bien chez les mâles que chez les femelles. Les niveaux les plus élevés sont observés dans le cortex des mâles et des femelles, tandis que les niveaux les plus faibles sont retrouvés dans le cervelet. Le TBT induit également une augmentation des niveaux de glutathione antioxydante (GSH) dans le striatum, l'hippocampe et dans les structures corticales mais pas dans le cervelet où les niveaux de TBT sont plus faibles. Nos découvertes indiquent que le TBT modulait les systèmes immunitaire et nerveux causant des perturbations endocrinienne et nerveuse.

Toxicological & Environmental Chemistry
Vol. ??, No. ?, Month?? 2009, 1–19

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Toxicological effects of mouse diet contaminated with tributyltin on the immune and neurological systems of C57BL/6 mice

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(*Received 13 November 2008; final version received 2 June 2009*)

Organotin compounds, widely used as antifouling agents, are known to bioaccumulate in the food chain. Among organotin compounds, tributyltin (TBT), a toxic and widespread contaminant, has become a serious factor in environmental pollution and is suspected of being immunotoxic in animals. The purpose of this study was to assess the effects of 80 μg of TBT per kilogram of body weight (kg bw), provided in food, on the immune and neurological systems of C57Bl/6 mice. Data showed that TBT increased the proliferation of T and B lymphocytes in both adult and juvenile female mice and juvenile male, but that it decreased the proliferation of both T and B lymphocytes in adult male mice. The macrophages activity in female and male juvenile mice was higher than in adults of both sexes. The natural killer cytotoxic activity was also increased in juvenile and adult males and females compared to the control groups. In the brain, we observed the presence of TBT in the hippocampus, the striatum, the cortex, and the cerebellum in both the male and female groups. The highest levels were observed in cortex of females and males, while the lowest levels were found in the cerebellum. TBT also induced an increase in the levels of the antioxidant glutathione (GSH) in the striatum, the hippocampus and in cortical structures but not in the cerebellum where the levels of TBT are lower. Our findings indicate that TBT modulated the immune and nervous systems causing endocrine and nervous perturbations.

Keywords: TBT; mice; toxicity; immune system; nervous system

Introduction

Organotin compounds are organometallic chemicals widely used in agriculture and industry. One of these, tributyltin (TBT), is mainly used for slime control in paper mills, for wood preservation and for disinfection of circulating industrial cooling waters (Adeeko et al. 2003; Mansueto et al. 2003). However, the most extensive use of TBT is in antifouling paints used for coating structures exposed to the aquatic environment such as ships, oils rigs, pleasure boats, and water intake pipes; this results in the contamination of marine and freshwater environments. Due to its toxicity for aquatic organisms, many countries have restricted the use of TBT in

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ISSN 0277–2248 print/ISSN 1029–0486 online
© 2009 Taylor & Francis
DOI: 10.1080/02772240903092298
http://www.informaworld.com

antifouling paints (van Wezel and van Vlaardingen 2004). But high concentrations of organotin compounds are still detected in sea water and in the sediment of coastal areas (Lee, Hsieh, and Tien 2006; Murai et al. 2005). Monobutyltin (MBT) and dibutyltin (DBT) are byproducts and/or metabolites of TBT. Butyltins possess both lipophilic and ionic properties that promote bioaccumulation in lipids and binding to macromolecules upon exposure. Furthermore, organotin compounds can accumulate in the food chain and concentrate in tissues. Humans become exposed to these compounds through the ingestion of contaminated fish and shellfish.

Only very few data are available on the effects of organotin compounds on mammals. The toxicity of organotin compounds was thus demonstrated in laboratory animals (reviewed by the World Health Organization (WHO 1999)) and in humans (Heidrich, Steckelbroeck, and Klingmuller 2001). In experimental rodents, TBT produced immunotoxicity, reproductive toxicity, and neurotoxicity, showing the highest concentrations in the brain followed by the liver and fat (Omura et al. 2004). It is clear from these studies that the immune system is a target for TBT toxicity, rendering animals more vulnerable to infectious diseases. Several immunotoxic effects, such as a reduction in the weight as well as morphologic changes of lymphoid organs, especially the thymus, with peripheral T cell depletion and suppression of T cell-mediated response (Inadera and Shimomura 2005), reduced the number of circulating lymphocytes, reduced total serum immunoglobulin levels, and alterations in lymphoid function were observed in weaned rats which received high doses ($>3\,mg\,kg^{-1}$) of TBT $bw\,day^{-1}$ (Penninks 1993). Long-term feeding studies of rat offsprings daily with doses of 0.025, 0.25, and $2.5\,mg\,kg^{-1}\,bw\,day^{-1}$ resulted in a significant increase of the mean percentage and of the absolute number of natural killer (NK) cells in male and female rats treated with the high dose of TBT (Tryphonas et al. 2004). TBT induced apoptosis, *in vitro*, in a murine macrophage cell line (Nakano, Tsunoda, and Konno 2004). In spite of all these data, the mechanisms of immunotoxicity of TBT are still not clear. Nonetheless, some studies suggest that it is the major TBT metabolite, DBT, which is responsible for the thymic toxicity. The primary effect of DBT is to interrupt the maturation of developing thymocytes by inhibiting their binding to thymic epithelial cells (Pieters et al. 1995; Snoeij, Penninks, and Seinen 1988). In many studies, animals exposed to TBT have exhibited permanent brain damage most notably in the case of early postnatal exposure. TBT is also toxic to the developing nervous system (O'Callaghan and Miller 1988) and an exposure to TBT via placenta and dam's milk seriously affected the cerebral N-methyl D-aspartate (NMDA) receptors involved in memory and learning (Konno, Tsunoda, and Sugita-Konishi 2005). Exposure to TBT has been shown to induce glutamate release and subsequent activation of glutamate receptors, leading to neuronal death (Nakatsu et al. 2006). Liu et al. (2006) have found that TBT induced a dose-dependent increase in the level of reactive oxygen species (ROS) in livers of rats after 3 and 7 days of exposure to TBT. Consequently, it can be concluded that exposure to TBT can promote, *in vivo*, oxidative damage in mammals (Liu et al. 2006).

The development of the immune system does not stop in the early postnatal period. A continuous remodeling of primary and secondary immune organs in the mouse takes place throughout life, with more important changes before puberty such as a remarkable decline in the relative weight of the thymus, spleen, and lymph nodes occur, whereas after puberty only the thymus continues its involution (Dominguez-Gerpe and Rey-Mendez 1998). Dietert et al. (2000) have also identified two windows

of vulnerability for the developing mouse immune system: the first occurs immediately after birth and the second begins at day 30 and continues to sexual maturity (day 60). Administration of toxic chemicals to young animals may alter immune functions, in a more severe way than in adults; these may also persist for a longer period of time. For example, preweanling rats were more sensitive to the immunotoxic effects of bis(tri-*n*-butyltin) oxide (TBTO) exposure than adult rats (Smialowicz et al. 1989). This continuous development, associated with the paucity of age-related immunotoxicity data call for studies assessing the immunotoxic potential of toxicants at different ages and, with a particular emphasis around the time of puberty. In response to this need, some long-term feeding studies were carried out by Vos et al. (1984, 1990). These were based on a diet containing 50 mg of TBTO kg^{-1} of food (equivalent to 2.5 mg kg^{-1} bw day^{-1}) to weaned male rats, which resulted in a significant reduction of thymus weight as well as a reduced resistance to *Trichinella spiralis* infection. In older rats, on the other hand, the effects of TBTO (12-months old) following treatment for 5 months were less pronounced.

This study was initiated in response to the need for additional data regarding the potential health effects of butyltins. Among xenobiotics, we chose tributyltin chloride which is used primarily as a biocide to prevent the attachment and growth of marine organisms to ship hulls and fishing nets. In this study, we have investigated the adverse effects of TBT on different parameters of immune and nervous systems in adult and juvenile mice of both sexes.

Materials and methods

Animals

C57Bl/6 males (15 g: 4–6 weeks, 25 g: 8–10 weeks old) and females (12 g: 4–6 weeks, 20 g: 8–10 weeks old) mice were purchased from Charles River Canada (St. Constant, QC, Canada). They were placed in an exposure room with an automatic 12 h light/dark cycle and fed with a standard rodent diet and tap water *ad libitum* for the 2-week acclimatization period.

After this time, they were randomly distributed in two groups for each sex: control and treatment group. The mice were fed a 20% preparation of lyophilized salmoned trout mixed with AIN-76A powder (Research Diets, NJ, USA) for the control group and for the treated group the same preparation to which was added 80 μg kg^{-1} bw day^{-1} of TBT. This dose selected for this experiment was based on the calculated tolerable daily intake (TDI) of 0.25 μg kg^{-1} bw day^{-1} which included a safety factor of 100 (Penninks 1993). Thus, the dose we used represents 360 times the TDI value.

Chemicals

Tetramethylammonium hydroxide (TMAH), monobutyltin trichloride, dibutyltin dichloride, tributyltin chloride, and tripentyltin chloride, NADPH, 5-sulfosalicyclic acid, 1-chloro-2,4-dinitrobenzene, and 5,5-dithiobis(2-nitrobenzoic acid) were purchased from Sigma-Aldrich (ON, Canada) and dichlorofluorescein diacetate from Invitrogen (ON, Canada). Tetrapropyltin was obtained from Alfa Aesar (MA, USA), sodium tetraethylborate from Strem Chemicals, Inc. (MA, USA) and silica gel 60 from EMD Chemicals (ON, Canada). All solvents were of analytical grade.

Dose administered

The dose administered was 80 μg kg^{-1} bw day^{-1}. The TBT was administered three times a week in mice food containing 20% of lyophilized salmoned trout (Canada Changmin Co., QC, Canada) in AIN-76A diet. The dose of TBT selected for this study was based on the maximum concentration found in fresh fish in the St.-Lawrence Estuary (0.1–0.5 mg kg^{-1} of fresh fish). This was considered a realistic dose and was established from the environmental contamination data established by Michaud and Pelletier (2006).

Cell suspensions

After 1 month of experimentation, the mice were killed by CO_2 anoxia. The liver, thymus, spleen and brain were removed (aseptically for the spleen) and weighted. The brain was snap-frozen and stored at −80°C for further analysis. The spleen and the thymus were mashed in RPMI-1640 medium (Sigma-Aldrich, ON, Canada). The cell suspensions were then filtered through nylon wool into Pasteur pipettes, washed twice in RPMI, and then recovered by centrifugation at 250 × *g* for 10 min. Cells were suspended in RPMI-1640 supplemented with 10% (v/v) foetal calf serum (Sigma-Aldrich) 1% penicillin/streptomycin (Sigma-Aldrich) and 10 mM L^{-1} HEPES (Sigma-Aldrich) (Brousseau et al. 1998). Cell viability was determined by trypan blue exclusion under light microscopy.

Determination of lymphocyte proliferation

Suspension of 5×10^5 viable spleen cells was prepared in complete RPMI-1640 medium. Spleen cells were plated with predeterminated optimal concentrations of concanavalin A (Con A 2.5 μg mL^{-1}, Sigma) or of lipopolysaccharide (LPS 200 μg mL^{-1}, Sigma) in flat bottom 96-well plates (Sarstedt, QC, Canada) under sterile conditions. For the negative control, spleen cells were incubated with RPMI-1640 medium alone. Samples were tested in triplicate. The microplates were incubated in a humidified incubator set at 37°C with 5% CO_2 for 54 h; then the cultures were pulsed with 0.5 μCi of [^{3}H]-methyl thymidine (MP Biomedicals, CA, USA) for an additional 18 h. Cells were harvested with a semi-automatic cell harvester (Skatron Instruments, Lier, Norway) and transferred on fiber-glass filter. Incorporated radioactivity was determined using a RackBeta from LKB (Turku, Finland) scintillation counter.

Determination of macrophage phagocytic activity

Phagocytosis was assessed by determining flow cytometry, the ingestion of fluorescent latex beads (Polysciences, ON, Canada) by phagocytes. Macrophages were cultured in two replicates per treatment group at 5×10^5 cells per tube. They were incubated under constant light agitation at 37°C for 90 min (optimal time for assay) with fluorescent latex beads at an approximate ratio of 100 beads/cell. At the end of the incubation period, the cells were layered over a 3% bovine serum albumin (BSA) (MP Biomedicals, CA, USA) gradient and centrifuged at 150 × *g* for 8 min at 4°C to remove free beads or beads which adhered to the surface of the cells. The cell pellets were then re-suspended in 0.5 mL of 0.5% phosphate-buffered formalin

(Sigma-Aldrich), and the number of engulfed beads was determined using a FACSCalibur flow cytometer (BD, NJ, USA). Analyses were done using two markers defined as the phagocytosis and then as the activity of phagocytes.

Determination of NK cell activity

The NK cell activity was measured using the flow cytometry assay (Brousseau et al. 1998). Briefly, a cellular suspension of YAC-1: target cells (ATCC, VA, USA) was adjusted 10^7 cells mL^{-1} and stained with 3,3′-dioctadecyloxacarbocyanine perchlorate (DIO) (Sigma) at 37°C with 5% CO_2 for 20 min. After the incubation, cells were washed twice and adjusted to 10^6 cells mL^{-1}. In 5 mL round bottom polypropylene tubes (Sarstedt), 20 μL of labeled YAC-1 tumor cells were added to 800 μL of viable spleen cells previously adjusted to 10^6 cells mL^{-1} with an effector: target ratio of 40 : 1. Cells were centrifuged at 150 × *g* for 5 min, protected from light and incubated in a humidified incubator at 37°C with 5% CO_2 for 4 h. 15 μL of propidium iodide (PI) (1 mg mL^{-1}) (Sigma) were added to each tube, 10 min before the readings. Cells were analyzed using a FACSCalibur flow cytometer and results were expressed as percentage of lysed target cells (YAC-1) compared to the control.

Determination of TBT in different parts of the brain

Extraction and derivatization

Because samples to be analyzed were less than 50 mg, the extraction method usually applied to organotins analysis in biological tissue, (Viglino, Pelletier, and Lee 2006) had to be modified. Briefly, the first step was basic digestion of the sample with TMAH, followed by simultaneous extraction with an organic solvent and ethylation of ionic butyltin compounds with sodium tetraethylborate. Complete treatment of the sample was performed in a 1.5 mL microcentrifuge tube (Eppendorf). The complete protocol was conducted several times without sample to make sure that polypropylene could resist the chemicals.

The details of the procedure were as follows: to about 20 mg of wet tissue 200 μL of TMAH was added, the tube was capped and shaken on vortex for 10 s. The tubes were then placed in a sonic bath for 60 min at 60°C. Once returned to room temperature, the digest received 500 μL of acetate buffer (pH = 4.1, 1 M L^{-1}), 50 μL of a 4 μg mL^{-1} solution of tripentyltin chloride as a surrogate for recovery correction, 400 μL of a toluene : hexane mix (1 : 1) and 100 μL of an aqueous solution of sodium tetraethylborate at 2% (prepared daily). The tube was shaken on vortex for at least 30 s and 15 μL of the internal standard tetrapropyltin (5 μg mL^{-1} in hexane) was added. The tube was centrifuged at 2600 × *g* for 6 min. A volume of 100 μL of the organic phase was withdrawn and cleaned on a home-made silica gel column topped with sodium sulfate; elution was done with 3 mL of toluene : hexane mix (1 : 1) and the final volume of the organic extract was reduced to 200 μL, in a precision glass tube, under a gentle stream of N_2 at room temperature before injection.

Detection and quantification of ethylated butyltins

Separation, detection and quantification of the volatile ethylated butyltins were performed by gas chromatography coupled to mass spectrometry

(Trace GC–Polaris Q, Thermo). The column on the GC was a DB5-MS, 30 m × 0.32 mm, from J&W Scientific, with a helium flow of 1.5 mL min^{-1}. The program temperature of the GC was as follows: initial temperature of the oven was 70°C for 4 min, increased to 250°C at 12°C min^{-1} and held for 2 min before a final increase to 290°C at 30°C min^{-1}, the final temperature was maintained for 10 min. The injector was at 250°C. The mass spectrometer (ionic trap) was operated in the SIM mode with detection of positive ions following electronic impact ionization (EI, 70 eV). Quantification of the different species of butyltins was performed with calibration curves relating signal ratio of the analyte to internal standard with concentration expressed as pg Sn μL^{-1}. Five points were used with concentrations ranging from 4 pg Sn μL^{-1} to 78 pg Sn μL^{-1}. The lowest value corresponded to a concentration of 160 ng Sn g^{-1} in a tissue sample of 0.02 g. Performance of the method was assessed by triplicate analysis of mussel tissue sample spiked with MBT, DBT, and TBT. The standard deviations (SDs) were 4.3%, 17.3%, and 30.7% for TBT, DBT, and MBT, respectively. Recovery ranged between 90% and 100% for TBT and DBT, and 70% and 100% for MBT. Recovery of the surrogate (tripentyltin chloride) was 95–100% in test samples and real samples, thus correction of analyte concentrations was not necessary.

Preparation of homogenates from brain structures

The following structures of the brain were removed: hippocampus, striatum, cerebral cortex, and cerebellum. Briefly, samples were homogenized in phosphate buffer (25 mM L^{-1}, pH 7) and centrifuged (1000 × *g*, 10 min, 2°C). GSH, H_2O_2, ROS, and GST were analyzed in the supernatant.

Determination of glutathione levels in different parts of the brain

Total glutathione (GSH) was determined according to Anderson (1989). Briefly, samples were homogenized in phosphate buffer (25 mM L^{-1}, pH 7) and centrifuged (1000 × *g*, 100 min, 2°C). They were then deproteinized with 5-sulfosalicyclic acid (3% w/v) during 15 min at 0°C. The suspension was then centrifuged (5 min, 14,000 × *g*). Then 100 µL of supernatant containing brain homogenates were incubated with 0.1 mL of 6 mM L^{-1} of 5.5-dithiobis(2-nitrobenzoic acid) (Sigma) and 0.8 mL of 0.143 M L^{-1} sodium phosphate buffer (pH 7) containing NADPH (0.248 mg mL^{-1}) and 6.3 mM L^{-1} EDTA (pH 7.5). The reaction was initiated by the addition of 2.6 units of GSH reductase. The rate formation of 5-thio-2-nitrobenzoic acid at 412 nm during 2 min, at 30°C, is proportional to the total GSH concentration. Values for total GSH were calculated from a standard curve.

Quantitative determinations of H_2O_2 by electrochemical detection

The APOLLO 4000 Free-Radical Analyzer (World Precision Instruments Inc., FL, USA) was used to monitor in real-time the level of H_2O_2. A pulse voltage maintained on a sensitive and selective H_2O_2 sensor (ISOHOP2) ensured that the electrochemical responses (redox current) generated at the working electrode were derived only from the oxidation of H_2O_2. Thereby these responses were proportional to the concentration of H_2O_2. To this end, the electrode was allowed to equilibrate for 15–20 min

in phosphate buffer that was stirred continuously by a magnetic agitator. Calibration curves for the H_2O_2 electrode were established prior to and following all tests. The latter was obtained by plotting changes in current (pA) against changes in H_2O_2 concentration.

Determination of the levels of ROS levels by 2′,7′-dichlorodihydrofluoresceine diacetate fluorescence in different structures of the brain

The dichlorodihydrofluoresceine diacetate (H_2DCF-DA) fluorescence procedure has been described elsewhere (Bastianetto et al. 2000; Behl et al. 1994). Briefly, 10 μL^{-1} of (H_2DCF-DA) was incubated with homogenates at 37°C. Upon oxidation, the nonfluorescent compound 2′,7′-H_2DCF-DA is converted to the fluorescent 2′,7′-H_2DCF-DA. The intensity of the fluorescence was measured in a microplate reader at with λ_{ex} at 495 nm and λ_{em} at 520 nm.

Determination of glutathione transferase activity in different parts of the brain

Glutathione transferase (GST) activity was measured in a 96-well plate reader, with the reaction mixture consisting of proteins samples (30 μg), 0.1 M L^{-1} phosphate buffer (pH 7.4) with EDTA 1 mM L^{-1}, a mix of GSH 1 mM L^{-1} and 1-chloro-2,4-dinitrobenzene (CDNB) in a total volume of 200 μL according to Habig, Pabst, and Jakoby (1974). Changes in absorbance were recorded at 340 nm for 5 min at 25°C. The specific GST activity is defined as 1 μM L^{-1} of 1-chloro-2,4-dinitrobenzene conjugate formed per minute per milligram protein.

Statistical analysis

Data are presented as the mean ± SD. The results were tested for homogeneity using an analysis of variance (ANOVA). Significant differences between treated and control groups were determined by Holm-Sidak test using the SigmaStat 2.03® from Jandel Scientific Software (San Rafael, CA, USA). Body weights were analyzed using two-way repeated measures ANOVA for age/sex and dose. Differences between treatment means were considered significant at $p < 0.05$.

Results

Body weights

Exposure to TBT had no significant effect on the mean body weight of any of the groups, regardless of sex or age, in either of the experiments (data not shown).

Determination of lymphocyte proliferation

Immunocompetent cells require continued proliferation and differentiation for self-renewal and for the protection of the host against pathogens. One way to evaluate their activity is the lymphoproliferative response of B and T cells to mitogens. We noted a positive proliferation in T and B cells in both young and adult female groups and for young males. However in adult males, the proliferation for T and B cell

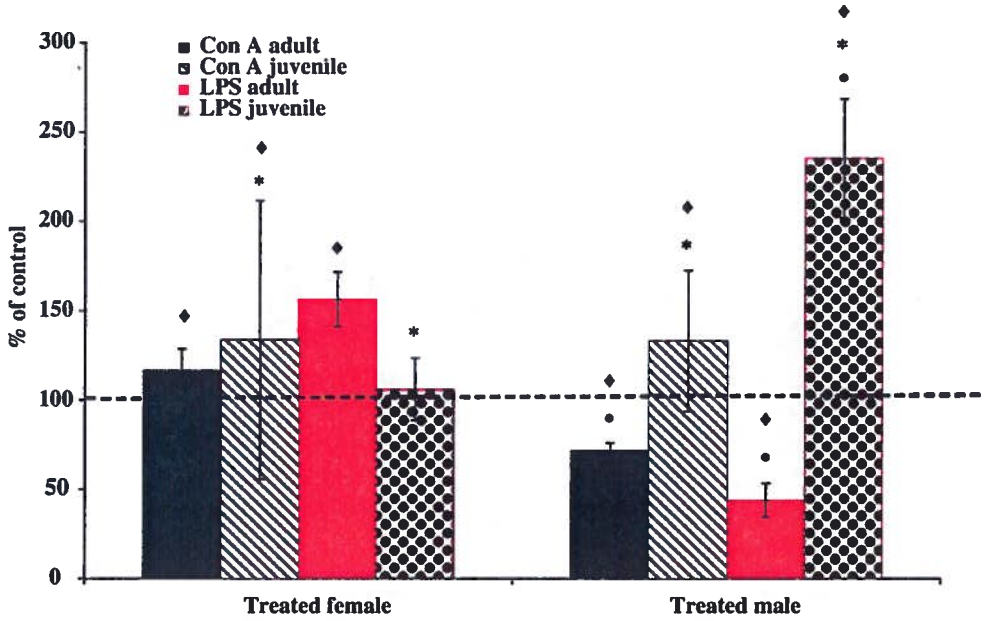

Figure 1. Determination of lymphocyte proliferation of treated females and males, adult and juvenile with Con A and LPS mitogens. The control level (100%) represents results from females and males, adult and juvenile without contamination by TBT. *: Denotes a significant difference compared between adult and juvenile groups with $p<0.05$; •: Denotes a significant difference when compared to male and female groups with $p<0.05$; ♦: Denotes a significant difference when compared to the control group with $p<0.05$.

was negative. Moreover, the proliferation for T cells in juvenile females (+34) was significantly higher ($p<0.05$) than the corresponding proliferation in adult females (+16). In adult females, the proliferation for B cells (+56) was significantly different ($p<0.05$) from that in juvenile females (+6). The T lymphocyte proliferation in juvenile males (+33) was significantly higher ($p<0.05$) than in adult males (−29). The same goes for the B cell proliferation in juvenile males (+135) which is significantly higher ($p<0.05$) than the corresponding proliferation in adult males (−56). The proliferation for T lymphocytes in contaminated adult females (+16) is significantly higher ($p<0.05$) than the corresponding proliferation in adult males (−29). The same is true for the B cell proliferation in adult contaminated females (+56). However, the proliferation for B cells of contaminated young females (+6) is significantly lower ($p<0.05$) than for contaminated young males (+ 135) (Figure 1).

Determination of macrophages phagocytic activity

The first line of defense in invertebrates and vertebrates is phagocytosis of foreign substances. Macrophages have an immunological role as the first line of cellular defense.

The phagocytosis and the activity of phagocytes in juvenile males and females were significantly higher ($p<0.05$) than the same parameters in adult males and females. However there was a negative phagocytosis and activity of phagocytes in females. The phagocytosis and the activity of phagocytes in males (adults and juveniles) were higher than in females (Figure 2).

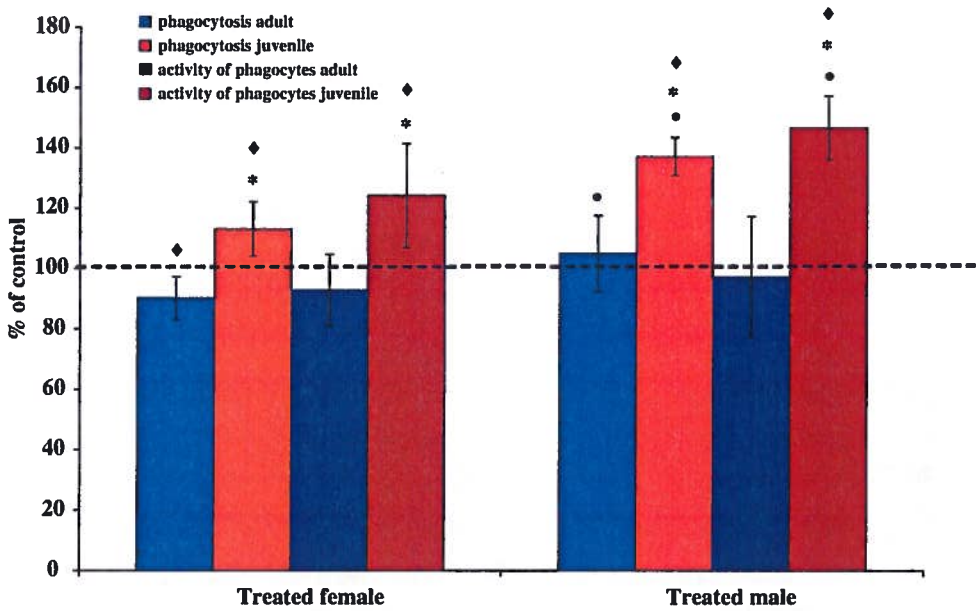

Figure 2. Determination of macrophage's phagocytic activity of treated female and male, adult and juvenile. The control level (100%) represents results from females and males, adult and juvenile without contamination by TBT. *: Denotes a significant difference compared between adult and juvenile groups with $p<0.05$; •: Denotes a significant difference compared between the male and female groups with $p<0.05$; ♦: Denotes a significant difference when treated groups are compared to control group with $p<0.05$.

Determination of NK cell activity

NK cells play a role in tumor resistance, host immunity to viral infections, and in the regulation of lymphoid and other hematopoietic cell populations. We noted a positive percentage of lysis in all groups except in juvenile females. Moreover the percentage of lysis of males was higher than of females (in both adult and juvenile groups) (Figure 3).

Determination of TBT concentrations in different structures of the brain

We analyzed the concentrations of TBT also in various parts of the brain (Figure 4), such as the striatum (ST), the hippocampus (H), the cortex (TCx), and the cerebellum (Cb). We noticed similar curves for both male and female groups, where we have a high concentration of TBT for male in the cortex (501 ng Sn g^{-1} tissue) and for female in the striatum (291 ng Sn g^{-1} tissue). For all tins analysis: due to the constraint of a large amount of protein needed for the assay, each section of the brain was treated only once. Hence there is not a calculated SD. However, the results obtained are clearly above the detectable limit(s) of the nontreated control samples.

Determination of GSH levels in different structures of the brain

We analyzed the concentrations of GSH in striatum (ST), hippocampus (H), cortex (TCx), and cerebellum (Cb) in control and treated groups at 8–10 weeks old

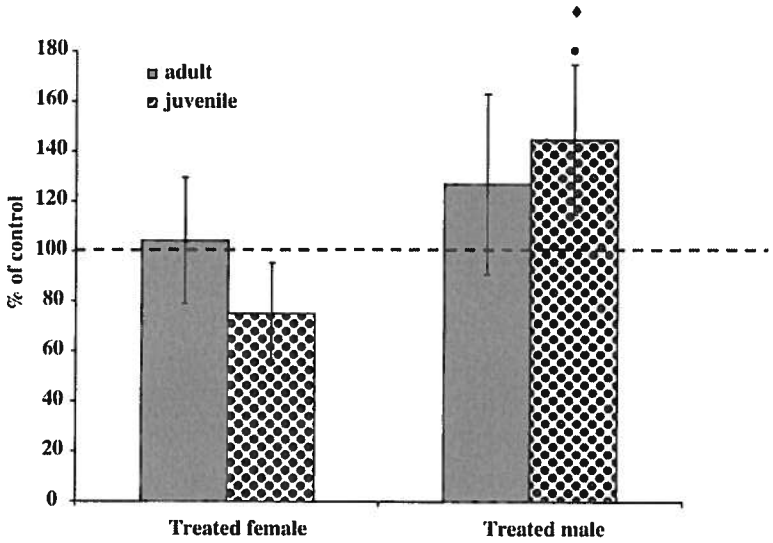

Figure 3. Determination of NK cell activity of treated females and males, adult and juvenile. The control level (100%) represents results from females and males, adult and juvenile without contamination by TBT. ●: Denotes a significant difference between male and female groups with $p<0.05$; ♦: Denotes a significant difference when treated groups are compared to the control group with $p<0.05$.

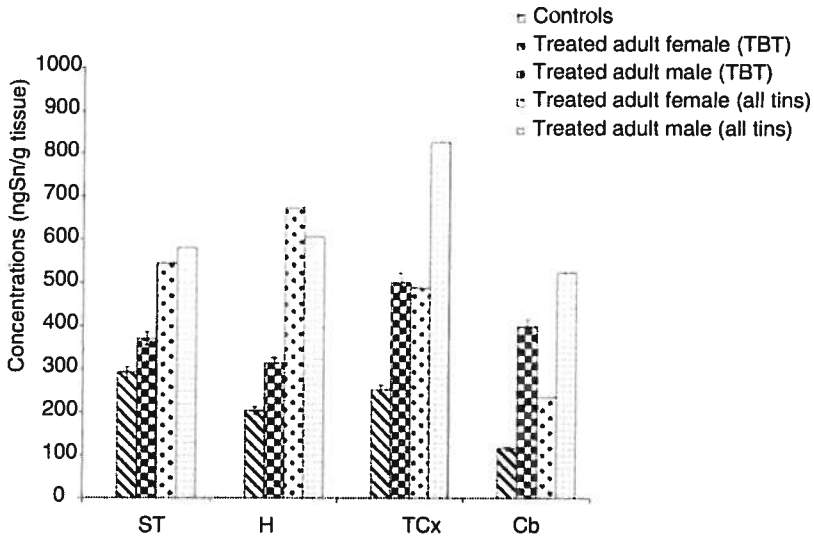

Figure 4. All TBT concentrations in different structures of brain (ST – striatum, H – hippocampus, TCx – cortex, Cb – cerebellum).

335 (adult) (Figure 5). We observed a significant increase ($p<0.05$ and $p<0.001$) of the GSH value in the treated group compared to the control group in different structures and for both sexes except for the cerebellum where we observed a significant decrease ($p<0.05$) of GSH levels in the treated groups.

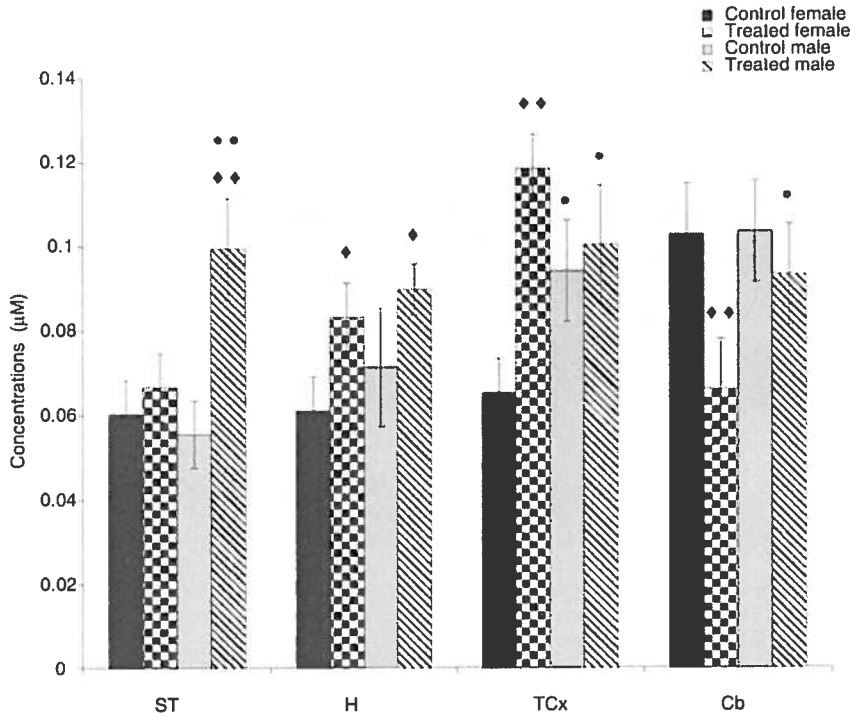

Figure 5. Concentrations of GSH, in different structures of the brain (ST–striatum, H–hippocampus, TCx–cortex, Cb–cerebellum) for control and treated groups at 8–10 weeks old (adult). ◆: Denotes a significant difference when treated groups are compared to control group with $p<0.05$, ◆◆ with $p<0.001$; •: Denotes a significant difference compared between the male and female groups with $p<0.05$, •• with $p<0.001$.

Determination of hydrogen peroxide and ROS levels in the brain

One of the main roles of the antioxidant GSH is to degrade H_2O_2. Therefore, in the presence of elevated GSH, we analyzed the level of H_2O_2 by electrochemical detection. We found that after the addition of 250 nM L^{-1} and 500 nM L^{-1} of H_2O_2, the pAs were 640 and 1291, respectively. After the addition of homogenates from cortex of control mice, the pA was 440 whereas it was 231 in the presence of homogenate from TBT-treated mice. The results obtained with homogenates from structures of the brain from different groups (Table 1) show that the levels of H_2O_2 were lower in TBT-treated group compared to control group except for the cerebellum. Therefore, in the presence of TBT in brain, the levels of GSH increased while the levels of H_2O_2 decreased in the hippocampus, the striatum and the cortex. These results prompted us to analyze the total level of ROS (by H_2DCF-DA fluorescence) in different structures of the brain. Our results (Figure 6) show that TBT treatment did not increase the levels of ROS.

Determination of GST activity in different structures of the brain

GST is an important drug-metabolizing enzyme that catalyzes the conjugation of reduced GSH with a variety of xenobiotic compounds. The presence of TBT in the

Table 1. Determination of hydrogen peroxide levels in homogenates from different structure of the brain by electrochemical detection.

	Male Control (pA*)	Male TBT (pA)	Female Control (pA)	Female TBT (pA)
Hippocampus	nd	nd	144	0
Striatum	957	235	310	108
Cortex	440	231	324	249
Cerebellum	286	364	259	373

Note: *pA: pico-ampere (10^{-12}). The concentration of H_2O_2 was obtained by plotting the intensity of the output signal in pA against the initial concentration of H_2O_2.

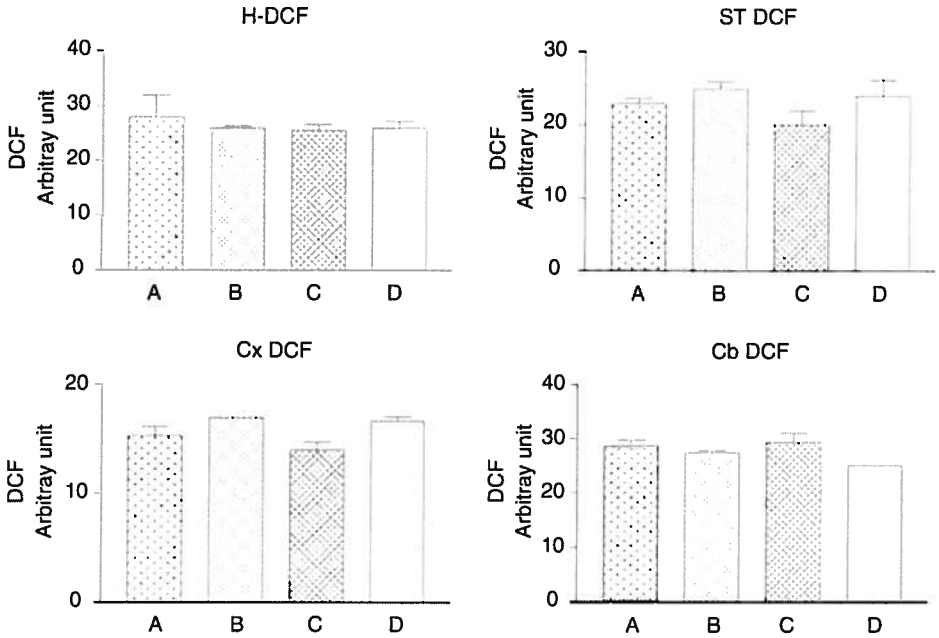

Figure 6. DCF levels in different structures of brain (ST – striatum, H – hippocampus, Cx – cortex, Cb – cerebellum). Legend: A – control female, B – treated female, C – control male, D – treated male.

brain prompted us to analyze the activity of GST in different structures of the brain. Our results demonstrate that GST activity is not modified by TBT treatment (Figure 7).

Separation, detection and quantification of the volatile ethylated organotins

360 The chromatogram of an extract from the cortex (of samples) of a contaminated mouse is shown in Figure 8. TBT and DBT are clearly identified, together with the surrogate, however no MBT was detected.

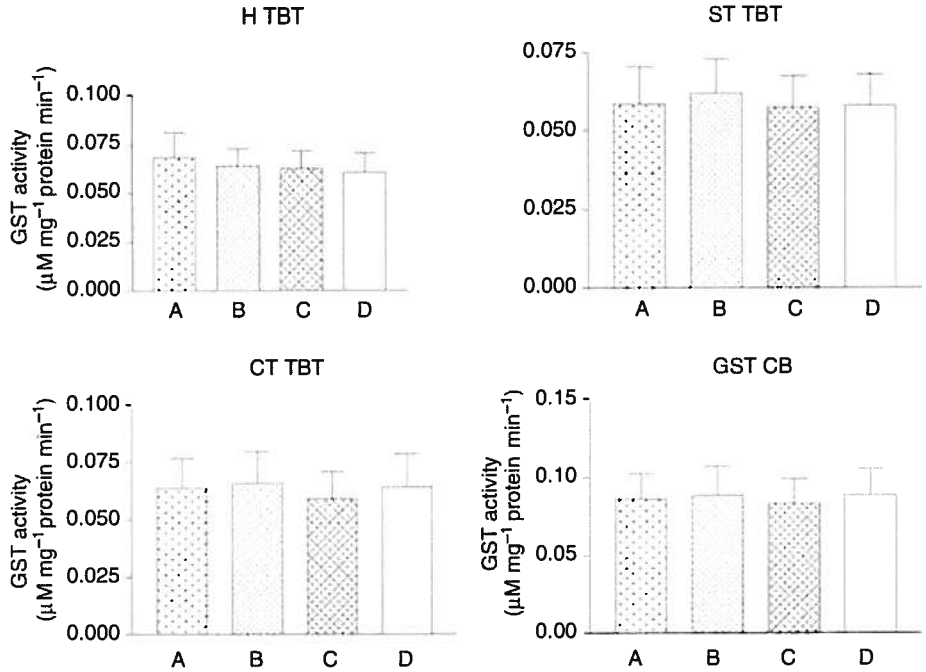

Figure 7. Glutathion-transferase activities in different structures of brain (ST – striatum, H – hippocampus, CT – cortex, CB – cerebellum). Legend: A – control female. B – treated female, C – control male, D – treated male.

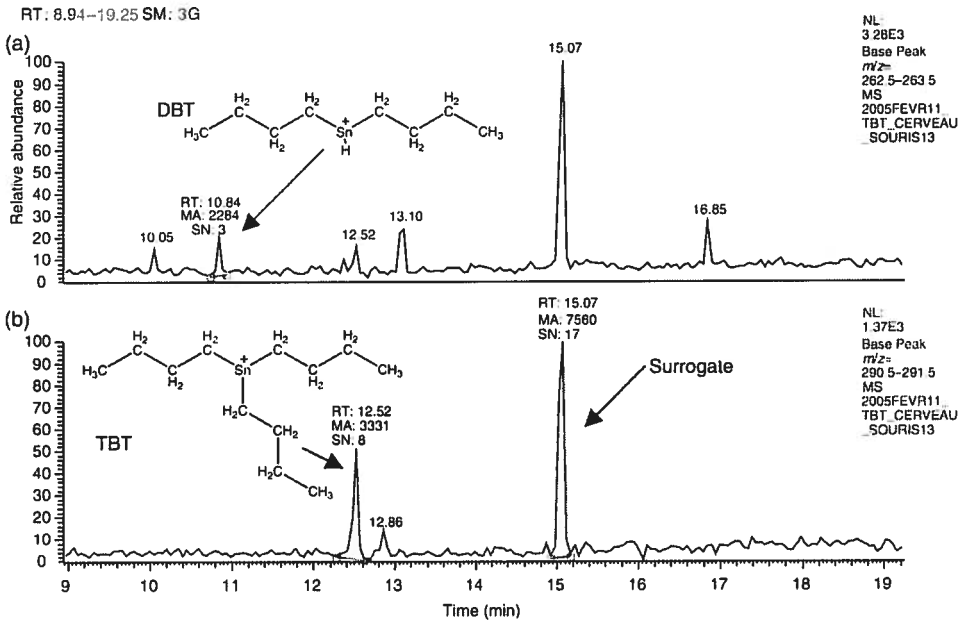

Figure 8. Sample CT-C pool (11–12–13). (a) Trace chromatogram for $m/z = 263$; (b) trace chromatogram for $m/z = 291$.

Table 2. Weights of different organs between each sex and groups.

		Spleen (g)	Thymus (g)	Brain (g)
Adults	Control female	0.09 ± 0.02	0.07 ± 0.02	0.02 ± 0.01
	Control male	0.10 ± 0.03	0.07 ± 0.02	
	Treated female	0.08 ± 0.01	0.06 ± 0.02	0.04 ± 0.02◆
	Treated male	0.09 ± 0.03	0.06 ± 0.02	
		Spleen	Thymus	Liver
Juveniles	Control female	0.10 ± 0.02	0.08 ± 0.02	1.26 ± 0.13
	Control male	0.12 ± 0.03	0.06 ± 0.02	1.90 ± 0.26
	Treated female	0.09 ± 0.01	0.07 ± 0.02	1.55 ± 0.20◆
	Treated male	0.08 ± 0.02◆	0.05 ± 0.02	1.95 ± 0.25

◆Denotes a significant difference when treated groups are compared to the control group with $p < 0.05$.

Weights of spleen, thymus, liver, and brain

Different organs of both sexes and groups (control and treated) were weighed (Table 2). For spleen and thymus, we observed a slight decrease in the weights of treated group compared to the corresponding control. The weight of brain was significantly increased ($p < 0.05$) in the treated female group compared to the control; we have no data for the other groups. For the liver we observed an increase for the treated juvenile group compared to the control.

Discussion

In this study, we investigated the adverse effects of TBT on different parameters of immune and nervous system in the feeding of the TBT-containing mouse food in mice of both sexes (male and female) and different ages (adult and juvenile). We found that TBT induced a positive proliferation for T and B lymphocytes except in adult males. This is in contrast to previous studies in mammals which suggested that lymphocyte proliferation was not affected by TBT exposure. Tryphonas et al. (2004) observed that there was no effect on the lymphocyte proliferation determined in the 60-day-old female rats and in the 90-day-old male rats. The results of our *in vivo* experiment showed an apparent increase in the sensitivity of lymphocyte proliferation suggesting than the humoral components of the immune system may be more affected by exposure to this chemical. Our observations reported differential responses of the lymphocyte populations from males to females on exposure to TBT: a significant inhibition of lymphocyte transformation in adult males and a significant activation of lymphocyte transformation in adult females. Thus, the lower T lymphocyte proliferation in males compared to females may be due to an increase of testosterone concentrations, since testosterone has been reported to increase apoptosis in T cells (McMurray et al. 2001). The same explanation applies to B lymphocyte proliferation since recent studies have shown an effect of estrogens on B cell development (Grimaldi et al. 2002). In fact, estrogens increase bone marrow progenitor B cells in mice by protecting the progenitor cells from apoptosis (Grimaldi et al. 2002; Hofmann-Lehmann, Holznagel, and Lutz 1998; Medina, Strasser, and Kincade 2000) and increase the survival in splenic B cells (Grimaldi et al. 2002).

Monocytes and macrophages play pivotal roles in the initiation of cellular and humoral immune responses. Macrophages have an immunological role as first line of cellular defense. The decreasing effect of oestrogen and progesterone on monocyte numbers may be due to sex hormones inducing mitotic arrest and apoptosis in monocytes (Thongngarm et al. 2003). In accordance to the findings of Raffray and Cohen (1993), juvenile male rodents showed an increase in the incidence of mononuclear phagocytic cells. These authors suggested that TBT acts directly on thymic macrophages causing either abnormal activation of phagocytosis, or a reduced clearance of apoptotic thymocytes derived from physiological cell deletion processes, but this possibility seems unlikely. Rice and Weeks (1989, 1990, 1991) demonstrated that the oxidative burst activity of toadfish peritoneal macrophages is extremely sensitive to TBT. At low doses, this compound activates macrophages in the absence of pharmacological stimulator. At higher concentrations, TBT is cytotoxic to phagocytes.

Previously published studies suggest that NK cells are sensitive to organotins. Thus, Vos et al. (1984) reported that exposure of male SPF-derived Wistar rats to 80 ppm of TBTO in the diet suppressed NK cell activity and Whalen, Loganathan, and Kannan (1999) showed that *in vitro* exposure of human peripheral blood lymphocytes for 24 h to 50–200 nM L^{-1} TBT suppressed cytotoxic activity from 40 to more than 90%. The significant enhanced NK cell activity in juvenile males reported in the present study is in contrast to the previously reported reduction in NK cell activity in both *in vivo* and *in vitro* exposure schemes to TBT and TBTO (Ghoneum et al. 1990; Smialowicz et al. 1989; Van Loveren et al. 1990; Whalen, Loganathan, and Kannan 1999). However, Tryphonas et al. (2004) observed also that TBT enhanced the mean NK cell activity in male rats. The percentages of B and NK cells were shown to be higher in newborn than in adult's humans (Erkeller-Yuksel et al. 1992). This could explain the enhanced NK cell activity we found in juvenile males. There are also indications that NK activity increases with age, but that on a per-cell basis their cytotoxic activity is diminished (Solana and Mariani 2000).

In the brain, in contrast to the liver, the distribution of TBT was shown to be higher than its metabolites MBT and DBT (Omura et al. 2004). These results prompted us to determine the levels of TBT in different structures of the brain involved in neurodegenerative diseases such as the hippocampus, the striatum, and the cortex. The cerebellum is usually considered as a control structure for these diseases. Moreover, as TBT could induce oxidative damages, the hippocampus and the striatum were selected in this study as both structures are well known to be more vulnerable to oxidative stress. TBT could also inhibit various parameters of cholinergic activity in the cortex area (Kobayashi et al. 1996) and the administration of TBT could decrease the concentration of serotonin in the cerebellum or the striatum (Tsunoda et al. 2006). Finally, DNA fragmentation was elevated in the cortex and the hippocampus following exposure to DBT (Jenkins, Ehman, and Barone 2004). Our results showed the presence of TBT after a chronic treatment in the hippocampus, the striatum, the cortex, and the cerebellum. Highest levels were observed in the hippocampus and the cortex in females and males, respectively while the lowest levels were observed in the cerebellum. These results are of great interest as we showed that the distribution of TBT in the brain is not correlated with that structure principally affected by lesion in neurodegenerative diseases.

Since TBT is known to induce oxidative stress (Liu et al. 2006; Liu and Xu 2007), we analyzed the effects on GSH levels of an administration of TBT. Our results

demonstrated that the administration of TBT induced an increase in the levels of GSH in the striatum, the hippocampus, and in cortical structures but not in the cerebellum where the concentration of TBT was lower. The higher resistance of cerebellum against TBT remains to be investigated. However, these results are consistent with a previous study demonstrating that TBT exposure reduced the concentration of some neurotransmitters in whole brain except in cerebellum (Lam et al. 2000). In parallel, the levels of hydrogen peroxide decreased without elevation of the ROS levels as determined through dichlorofluoresceine. These results suggested that the elevation of GSH is sufficient to degrade hydrogen peroxide and to counteract ROS production in brain. In contrast to some previous studies (Liu et al. 2006; Liu and Xu 2007), we demonstrated that, in the brain, a long-term treatment of TBT induced compensatory mechanism(s) to attenuate the oxidative stress induced by TBT.

It has been shown that TBT is an inhibitor of GST activity in the liver at micromolar concentrations (Mosialou and Morgenstern 1990; Tahir, Guthenberg, and Mannervik 1985). Our data indicated that TBT has no effect on the activity of GST. More studies are necessary to determine if these concentrations are sufficient to induce neuronal damages as observed in cell culture models (Jenkins, Ehman, and Barone 2004).

Taken together, these results indicate a gender difference in the balance between the specific and nonspecific immune response (i.e. a more profound and higher state of excitation of the nonspecific immune response and a relative suppression of the cellular immune response of the specific immune system in males compared with females). Similar observations have been recently made in the human (Bouman et al. 2004). Moreover, it has been reported that significant amounts of TBT were found in organs such as brain, liver, and fat from rat after oral administration of organotins (Omura et al. 2004). The brain and the immune system, or the "supersystems", a term coined by Tada (1997), are the two major adaptive systems of the body.

Acknowledgments

This work was supported by a grant from Toxic Substances Research Initiative of Canada, Canada Research Chair in Immunotoxicology (M.F.) and Canada Research Chair in molecular Ecotoxicology (E.P.).

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Date: mar. 2009-06-02 09:54
À: Fortier, Marlene
Cc: toxenchem@uni-bayreuth.de
Objet : Toxicological & Environmental Chemistry - Decision on Manuscript ID GTEC-2008-0090.R1

02-Jun-2009

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Reviewer(s)' Comments to Author:

Reviewer: 1
Comments to the Author
I have checked the revised manuscript. It seems to me OK now for publication

Date: 24 Aug 2009
To: "Heloise Frouin" heloise.frouin@iaf.inrs.ca
From: Cynthia Baldwin cbaldwin@vasci.umass.edu
Subject: VETIMM-D-09-1520R2 Decision

Ms. No. VETIMM-D-09-1520R2
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With kind regards

Cynthia L. Baldwin
Editor US
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Date: 04.09.2009
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From: Charles Sheppard charles.sheppard@warwick.ac.uk
Subject: Your Submission

Ms. Ref. No.: MPB-D-09-00203R1
Title: Effects of individual polybrominated diphenyl ether (PBDE) congeners on harbour seal immune cells in vitro
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From: Charles Sheppard charles.sheppard@warwick.ac.uk
Subject: Your Submission

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Subject: Toxicology: Submission Confirmation
To: heloise.frouin@gmail.com

Ms. No.: TOX-09-832
Title: Toxic effects of various pollutants in 11B7501 lymphoma B cell line from harbour seal (Phoca vitulina)
Corresponding Author: Mrs. Heloise Frouin
Authors: Michel Fournier, Professor

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