UNIVERSITÉ DU QUÉBEC À RIMOUSKI

UTILISATION D'UN POISSON MODÈLE, *FUNDULUS HETEROCLITUS*, POUR ÉVALUER LE POTENTIEL EMBRYOTOXIQUE DES POLLUANTS ORGANIQUES PERSISTANTS ACCUMULÉS PAR L'ANGUILLE D'AMÉRIQUE (*ANGUILLA ROSTRATA*) DU LAC ONTARIO DE 1988 À 2008

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À tous ceux qui comptent pour moi et se reconnaîtront en lisant ceci

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RÉSUMÉ

Le recrutement des juvéniles de l'anguille d'Amérique (*Anguilla rostrata*) vers le lac Ontario (LO), Canada, a décliné drastiquement depuis les années 1980. Afin d'explorer la contribution possible à ce déclin du transfert maternel des polluants organiques persistants (POP), la présente étude mesure les variations temporelles de la toxicité de mélanges organiques complexes extraits d'anguilles capturées dans le LO en 1988, 1998 et 2008, chez les embryons de *Fundulus heteroclitus* exposés par injection intravitelline (IVi). Afin d'identifier les composés les plus toxiques accumulés par les anguilles et de caractériser leurs effets embryotoxiques, des embryons de *F. heteroclitus* ont été exposés par injection IVi à des doses sous-létales de plusieurs POP historiquement mesurés dans les tissus des poissons du LO, incluant certains composés apparentés aux dioxines (TCDD, BPC126, BPC77 et 2,3,4,7,8-PnCDF), des BPC non-coplanaires (BPC52 et BPC110) ainsi qu'à un mélange technique de BPC, l'Aroclor 1254.

Tous les composés apparentés aux dioxines ainsi que l'Aroclor 1254 ont causé des malformations crânio-faciales, une réduction dose-dépendante de la croissance larvaire et de la capacité de prédation sans altération de l'activité locomotrice de base, ainsi qu'une induction de l'activité de l'ethoxyrésorufine-*O*-dééthylase (EROD) chez les larves de *F. heteroclitus*. Aucune de ces réponses n'a été observée avec les BPC non-coplanaires. Les relations dose-réponse pour l'activité EROD pour la TCDD, le BPC126 et le 2,3,4,7,8-PnCDF avaient des pentes similaires et les potentiels toxiques relatifs (PTR) du BPC126 et du 2,3,4,7,8-PnCDF par rapport à la TCDD ont été estimés à 0.71 et 2.40, respectivement. Ils étaient respectivement environ 140 et 5 fois supérieurs aux facteurs d'équivalence toxique (FET) de référence de l'Organisation Mondiale de la Santé (OMS) pour ces deux composés chez les poissons, qui sont basés sur des données d'embryo-mortalité obtenues chez la truite arc-en-ciel (*Oncorhynchus mykiss*). Ces résultats suggèrent que les PTR sont spécifiques à chaque espèce et que les FET actuels du BPC126 et du 2,3,4,7,8-PnCDF pourraient sous-estimer leurs potentiels toxiques chez certaines espèces de poissons.

Les pentes des relations dose-réponse pour la capacité de prédation étaient plus prononcées pour le BPC126 et le 2,3,4,7,8-PnCDF par rapport à la TCDD, suggérant différentes voies d'effets. Le niveau d'expression de plusieurs gènes a également été mesuré par réaction en chaîne par polymérase quantitative (qPCR) à la suite d'expositions à des doses uniques de TCDD ou de BPC126 (1280 et 1250 pg g^{-1} de poids humide, respectivement) causant un même niveau d'induction EROD. Un patron de réponses différent a été observé entre le BPC126 et la TCDD : le BPC126 a semblé induire des réponses anti-oxydantes par l'intermédiaire du gène *sod2*, mais pas la TCDD. Cela suggère que ces deux composés agissent selon des mécanismes différents pour induire les altérations comportementales observées chez les larves de *F. heteroclitus*, et que le stress oxydant est un facteur clef dans le cas du BPC126.

Les extraits d'anguille de 1988 et 1998 ont été les plus toxiques, provoquant un patron de réponses embryotoxiques sous-létales similaire à celui observé chez des embryons de *F. heteroclitus* exposés aux composés apparentés aux dioxines précédemment cités ou à l'Aroclor 1254 : retard de croissance, malformations crânio-faciales, induction de l'activité EROD et réduction de la capacité de prédation des larves. Le potentiel toxique des extraits a décliné avec le temps : le seul effet significatif observé avec les extraits de 2008 était l'induction de l'activité EROD. Les autres POP mesurés dans les extraits d'anguilles (BPC non-coplanaires, PBDE et pesticides organochlorés) n'ont pas semblé être d'importants contributeurs à l'embryotoxicité observée. En raison de la combinaison de ses concentrations élevées et de son PTR élevé par rapport à la TCDD chez les jeunes stades de vie de *F. heteroclitus*, le BPC126 était probablement le plus important contributeur à l'embryotoxicité observée avec l'Aroclor 1254 et les extraits d'anguille. Néanmoins, comme cela a pu être observé avec l'Aroclor 1254, les EQT-TCDD dérivés des données chimiques, calculés à partir des concentrations de certains composés apparentés aux dioxines dans les extraits et de leurs PTR respectifs chez *F. heteroclitus*, ont surestimé la capacité des extraits à induire l'activité EROD, possiblement en raison d'interactions entre les différents POP.

La toxicité des mélanges organiques complexes de POP accumulés par les anguilles du LO a pu être sous-estimée en raison de plusieurs facteurs, incluant les pertes chimiques durant la préparation des extraits, ainsi que l'absence d'évaluation de leurs effets à long terme. En plus de différences interspécifiques en termes de sensibilité à la TCDD déjà documentées dans la littérature, nos résultats démontrent qu'il existe des différences interspécifiques de FET. Il existe donc une incertitude quant à la prédiction de la toxicitié d'un mélange complexe de composés apparentés aux dioxines chez *A. rostrata* à partir de données obtenues chez d'autres espèces. Dans l'ensemble, nos résultats supportent l'hypothèse selon laquelle la contamination du LO par les composés apparentés aux dioxines aurait représenté une menace pour la population de l'anguille d'Amérique en altérant des paramètres pertinents d'un point de vue environnemental, telle que la capacité de prédation des larves. Ces résultats soulignent l'importance de tester l'embryotoxicité des composés apparentés aux dioxines chez les jeunes stades de vie des différentes espèces d'anguilles, d'explorer les effets à long terme d'extraits organiques d'anguilles sur les jeunes stades de vie des poissons et de développer des biomarqueurs pour évaluer les altérations potentielles chez les jeunes anguilles collectées sur le terrain.

Mots-clés : Anguille d'Amérique, *Fundulus heteroclitus*, TCDD, dioxine, BPC, Aroclor, embryotoxicité, comportement, TEF, EROD.

ABSTRACT

Recruitment of American eel (*Anguilla rostrata*) juveniles to Lake Ontario (LO), Canada, declined significantly since the 1980s. To investigate the possible contribution of maternally transferred persistent organic pollutants (POPs) to this decline, this study measured temporal variations in the toxicity of complex organic mixtures extracted from LO eels captured in 1988, 1998 and 2008 to *Fundulus heteroclitus* embryos exposed by intravitelline (IVi) injection. To identify the most toxic compounds accumulated by eels and to characterize their embryotoxic effects, *F. heteroclitus* embryos were exposed by IVi injection to sublethal doses of various POPs historically measured in LO fish tissues, including dioxin-like compounds (DLCs) (TCDD, PCB126, PCB77 and 2,3,4,7,8-PnCDF), non-dioxin-like (NDL) PCBs (PCB52 and PCB110) and also to a complex mixture of PCBs, Aroclor 1254.

All DLCs as well as Aroclor 1254 caused craniofacial deformities, a dose-responsive reduction of larval growth and prey capture ability without alteration of basal locomotor activity, and induction of ethoxyresorufin-*O*-deethylase (EROD) activity in *F. heteroclitus* larvae. None of these responses was induced by the two NDL PCBs. The dose-response relationships for EROD activity for PCB126, 2,3,4,7,8-PnCDF and TCDD had similar slopes and the relative potencies (RePs) of PCB126 and 2,3,4,7,8-PnCDF to TCDD for EROD activity were estimated at 0.71 and 2.40, respectively. They were approximately and respectively 140- and 5-fold higher than the World Health Organization (WHO) TCDD equivalency factors (TEFs) of PCB126 and 2,3,4,7,8-PnCDF for fish, which are based on rainbow trout (*Oncorhynchus mykiss*) embryolethality data. These results suggest that relative potencies are species-specific and that the current ReP for PCB126 and 2,3,4,7,8-PnCDF underestimates their toxicity for some fish species.

The slopes of the dose-response relationships for prey capture ability for PCB126 and 2,3,4,7,8-PnCDF were steeper than for TCDD, suggesting different pathways of effects. Expression levels of several genes were also studied by quantitative real-time polymerase chain reaction (qPCR) following exposure to single doses of TCDD or PCB126 (1280 and 1250 pg g^{-1} wet weight, respectively) causing similar EROD induction. A different pattern of responses was observed between PCB126 and TCDD : PCB126 appeared to induce antioxidant responses by inducing *sod2* expression, while TCDD did not. It suggests that these two compounds act through different mechanisms to produce behavioral alterations in *F. heteroclitus* larvae, and that oxidative stress may be a key factor in the case of PCB126.

The 1988 and 1998 eel extracts were the most toxic, causing a pattern of sublethal embryotoxic responses similar to those reported in *F. heteroclitus* embryos exposed to previously cited DLCs or to Aroclor 1254 : stunted growth, craniofacial deformities, induction of EROD activity, and reduced predatory capacities of larvae. The potency of these extracts declined over time : the only significant effect of 2008 eel extracts was EROD induction. Other POPs

measured in eel extracts (NDL PCBs, PBDEs and organochlorinated pesticides) did not appear to be important agonistic contributors to the observed toxicity. Due to the combination of its concentrations and its high ReP compared to TCDD in *F. heteroclitus* early life stages (ELS), PCB126 was probably the most important contributor to the overall embryotoxicity observed with Aroclor 1254 or eel extracts. However, as observed with Aroclor 1254, the chemically derived TCDD-TEQs of eel extracts, calculated using measured concentrations of some DLCs and their RePs for *F. heteroclitus* ELS, overestimated their potency to induce EROD activity possibly due to interactions among POPs.

The toxicity of the complex mixtures of POPs accumulated by LO eels may have been underestimated as a result of several factors, including the loss of POPs during extracts preparation and the non-assessment of their potential long-term effects. In addition to the interspecific differences in terms of sensitivity to TCDD documented in the literature, our results demonstrates that interspecific differences also exist for TEFs. Thus, there is uncertainty in the prediction of the toxicity of complex mixtures of DLCs to *A. rostrata* from data obtained with other species. Overall, our results support the hypothesis that contamination of LO with DLCs may have represented a threat for the American eel population through ecologically relevant effects such as altered larval prey capture ability. These results prioritize the need to assess ELS toxicity of DLCs in *Anguilla* species, to investigate long-term or delayed effects of complex eel extracts to ELS of fish and to further develop biomarkers to assess potential effects in eel ELS collected in the field.

Keywords : American eel, *Fundulus heteroclitus*, TCDD, dioxin, PCB, Aroclor, embryotoxicity, behavior, TEF, EROD.

TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

INTRODUCTION GÉNÉRALE

L'anguille d'Amérique

Généralités et biologie

L'aire de répartition dans les zones d'eau douce de l'anguille d'Amérique *Anguilla rostrata* (LeSueur 1817) s'étend sur environ 50° de latitude (figure 1), mais principalement entre 20° et 50° N (Barbin et McCleave, 1997). Tout comme l'espèce européenne *Anguilla anguilla* (Linné 1758), elle est amphihaline, catadrome et sémelpare[1]. Son cycle vital est complexe, atypique et ponctué par des passages dans des milieux océaniques, côtiers, estuariens ou encore d'eau douce (figure 2). Il est entre autres marqué par une des plus longues migrations reproductrices observées en milieu marin. La reproduction, de type panmictique (Côté et al., 2013), a lieu entre la fin de l'hiver et le printemps en mer des Sargasses et ce aussi bien pour l'anguille d'Amérique que celle d'Europe. La ponte de chacune de ces deux espèces se chevauche dans le temps et l'espace bien qu'elle soit en moyenne quelques degrés de longitude plus à l'ouest et environ un mois plus tôt chez l'anguille d'Amérique (van Ginneken et Maes, 2005). La découverte de l'aire de ponte découle de la capture dans cette région de l'océan Atlantique des plus petits individus juvéniles recueillis (Schmidt, 1922). En effet, aucun adulte mature ou embryon n'ont encore été capturés en mer des Sargasses à ce jour (Feunteun, 2002; Tesch et Henderson, 1977). En captivité, Oliveira et Hable (2010) ont rapporté des tailles variant de 0.960 à 1.030 mm chez les œufs d'*A. rostrata*, un diamètre comparable à ceux des espèces européennes et japonaises (*Anguilla japonica*). L'évolution dans le temps de l'embryogénèse de l'espèce américaine semble comparable à celle des espèces européennes et japonaises, mais les auteurs ont noté des disparités dans le temps nécessaire aux embryons pour éclore : 38-45 h chez *A. japonica*, 48-50 h chez *A. anguilla* et 32-45 h chez *A. rostrata*

1. Se dit d'une espèce qui ne présente qu'un seul cycle de reproduction au cours de sa vie.

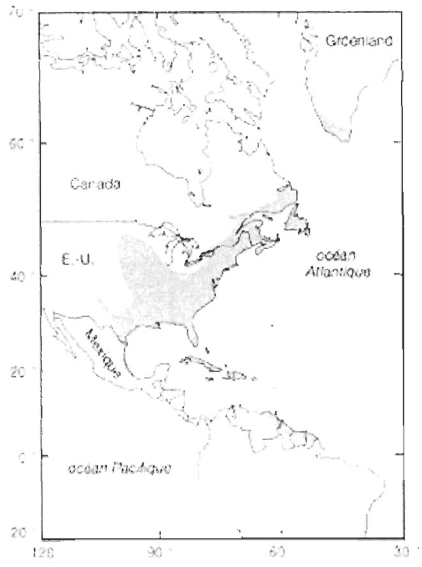

Figure 1: Aire de répartition de l'anguille d'Amérique. Adapté de Tesch et Henderson (1977).

(Oliveira et Hable, 2010). Ces disparités pourraient être dues à la qualité des œufs ou à la température d'incubation (Oliveira et Hable, 2010).

De forme aplatie, le leptocéphale représente la forme larvaire de l'espèce. Leur corps est transparent, car il est presque entièrement constitué de glycosaminoglycane (GAG) servant au stockage énergétique (Pfeiler et al., 2002) et exempt de globules rouges (Hulet et Robins, 1989), ce qui leur procure probablement une protection visuelle contre les prédateurs (Miller, 2009). À l'éclosion, le leptocéphale n'est que très peu développé. C'est le stade préleptocéphale : la larve ne présente pas d'yeux ou bien des yeux très peu développés, pas ou peu de dents lorsque la bouche est présente, ainsi qu'une unique gouttelette lipidique servant de ressource énergétique. À l'éclosion, Oliveira et Hable (2010) ont rapporté une taille égale à 2.7 ± 0.2 mm pour les préletocéphales d'*A. rostrata*. Le stade leptocéphale commence véritablement lorsque la gouttelette lipidique est totalement résorbée, que les yeux sont formés et la bouche présente (Miller, 2009). Le leptocéphale requiert alors une source d'alimentation externe pour survivre et se développer. À ce jour, la transition vers ce stade n'a

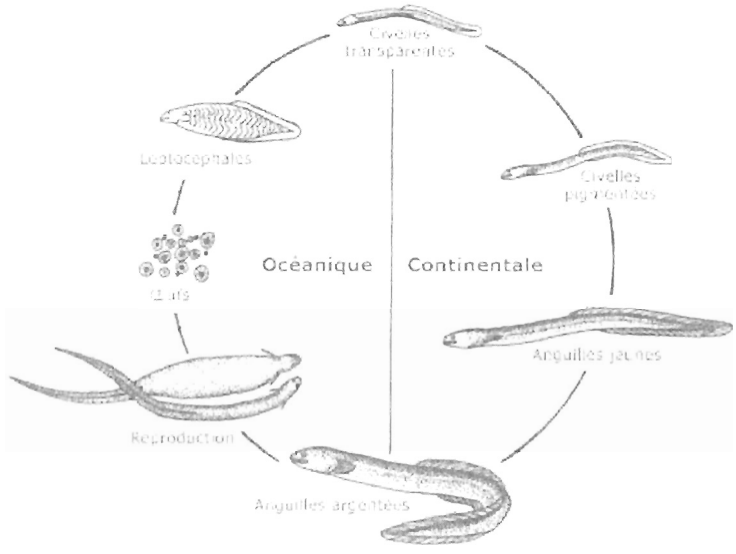

Figure 2: Représentation schématique du cycle vital de l'anguille d'Amérique (Ministère des Richesses Naturelles de l'Ontario, avec autorisation).

pas été décrite chez *A. rostrata*, Oliveira et Hable (2010) n'ayant pas réussi à faire survivre leurs larves au-delà d'un stade où la bouche était encore absente et la gouttelette lipidique presque totalement résorbée.

La biologie des leptocéphales est assez peu connue en raison des difficultés rencontrées pour les capturer et les maintenir vivants en captivité, notamment en raison de leur grande fragilité (Miller et Tsukamoto, 2004, 2006). Leur régime alimentaire est peu connu. Des études effectuées chez l'anguille japonaise suggèrent que les leptocéphales se nourrissent principalement de neige marine, de matière organique dissoute, de matières fécales de zooplancton ou encore de tests de Larvacés, mais pas directement de phytoplancton ni de zooplancton (Miller, 2009; Miller et al., 2013; Mochioka et Iwamizu, 1996; Otake et al., 1993). Néanmoins, des travaux récents utilisant l'ADN extrait du contenu du système digestif de leptocéphales de l'anguille européenne indiquent que ces derniers se nourrissent également d'une large variété de zooplancton gélatineux (Alfredsson, 2009; Riemann et al., 2010). Les leptocéphales des

anguilliformes grandissent jusqu'à une certaine taille maximale comprise entre 50 et 100 mm avant de se métamorphoser (Miller, 2009). Powles et Warlen (2002) ont rapporté des tailles d'anguilles juvéniles entrant dans les estuaires variant entre 53 et 61 mm en moyenne, à différentes stations s'étendant de la Caroline du Nord jusqu'au Nouveau-Brunswick. Kleckner et McCleave (1985) ont échantillonné des leptocéphales d'*A. rostrata* aussi grands que 70 mm. La taille des leptocéphales de l'anguille japonaise diminue légèrement au cours de la métamorphose (Tsukamoto et al., 2009), il est possible qu'il en soit de même chez l'anguille d'Amérique. À l'inverse d'autres larves de poissons, plusieurs études semblent indiquer que les leptocéphales ne consacrent pas l'essentiel de leurs dépenses énergétiques à leur croissance, mais plutôt à leur locomotion (Bishop et Torres, 1999; Bishop et al., 2000; Bishop et Torres, 2001). Les leptocéphales observés en aquarium sont de très bons nageurs (Miller et Tsukamoto, 2004). Cela peut aisément être déduit de la nécessité pour eux de se rendre aux aires de croissance (bien que les courants marins jouent là aussi un rôle) ainsi que la nécessité de fuir les éventuels prédateurs (Miller, 2009).

Le cerveau des leptocéphales est proéminent, et leurs systèmes oculaire et olfactif sont particulièrement bien développés (Pfeiler, 1989), la vision et l'olfaction étant leurs deux principaux sens (Smith, 1989). Il est intéressant de noter que la taille relative du globe oculaire diminue lors du passage aux stades juvéniles (Tomoda et Uematsu, 1996), suggérant que la vision tient un rôle important pour la capacité de prédation des leptocéphales. La présence unique de bâtonnets (sans cônes) dans la rétine de leptocéphales d'anguilles européennes va dans ce sens et suggère une adaptation aux conditions de faible luminosité (Pankhurst, 1984). Des leptocéphales d'*A. japonica* élevés en captivité ont une phototaxie négative à la suite d'un stimulus lumineux soudain (Yamada et al., 2009), ce qui coïncide avec leur distribution verticale diurne et nocturne dans le milieu naturel : on les retrouve généralement dans les premiers 100 mètres de profondeur pendant la nuit, et à environ 250-300 mètres de profondeur en plein jour (Castonguay et McCleave, 1987). Cette observation associée à la présence d'yeux adaptés aux conditions de faible luminosité suggère que les leptocéphales sont des prédateurs nocturnes. Les leptocéphales sont capables de se déplacer aussi bien vers l'avant

que vers l'arrière et de se contorsionner, tout comme les adultes (Miller, 2009). Ils possèdent une ligne latérale rudimentaire servant à la mécanoréception, telle que décrite chez *A. japonica* (Okamura et al., 2002), ce qui leur permettrait de réagir aux stimuli mécaniques générés par d'éventuels prédateurs (Fuiman et Magurran, 1994).

Les leptocéphales d'*A. rostrata* utilisent les courants de surface du Gulf Stream pour se rendre en direction du nord-ouest, vers les eaux côtières nord-américaines (Schmidt, 1922; Tesch et Henderson, 1977). La durée totale du stade leptocéphale est peu connue : elle serait comprise entre un et deux ans, voire moins, chez l'anguille d'Amérique contre deux à trois ans pour son homologue européen. Cette différence et la ségrégation entre les leptocéphales d'*A. rostrata* et *A. anguilla* qui en découle résulterait d'un taux de croissance plus élevé chez l'espèce américaine (Arai et al., 2000; Wang et Tzeng, 1998, 2000) mais également d'une distance de migration plus courte pour cette dernière. Une incertitude demeure quant à la durée exacte du stade leptocéphale de chacune des espèces, les différentes méthodes utilisées pour l'estimer pouvant donner des résultats très variables (Bonhommeau et al., 2010). Les leptocéphales d'*A. rostrata* atteignent les plateaux continentaux au cours de l'hiver ou du printemps (Martin, 1995; Tesch et Henderson, 1977) et y séjournent environ deux mois le temps de devenir des civelles pigmentées et d'amorcer leur passage en eau douce en transitant par les estuaires (Wang et Tzeng, 1998). Au préalable, les leptocéphales se métamorphosent en civelle transparente. La métamorphose est caractérisée par de nombreux changements morphologiques tels qu'un épaississement de la tête, une croissance du système olfactif et une perte des dents (Miller, 2009). On observe également une migration du système digestif, ainsi les leptocéphales ne se nourrissent pas durant la métamorphose (Otake, 2003). Les globules rouges et la pigmentation apparaissent progressivement pour laisser place au stade de civelle pigmentée (Miller, 2009; Otake, 2003). La salinité, la profondeur ou encore la composition chimique de l'eau sont autant de facteurs qui pourraient déclencher la métamorphose des leptocéphales (Otake, 2003). Il a été observé des leptocéphales d'*A. anguilla* se métamorphosant à des profondeurs de 1000 mètres en approche de l'Europe (Antunes et Tesch, 1997). Des civelles transparentes d'anguilles japonaises ont été observées très loin des côtes, suggérant

une métamorphose enclenchée bien avant l'arrivée aux plateaux continentaux (Otake et al., 2006). Le passage en eau douce des civelles pigmentées est déclenché et contrôlé en premier lieu par la température et le débit des estuaires, puis davantage par les cycles tidaux en fin de saison de migration (Edeline et al., 2006; Martin, 1995; McCleave et Kleckner, 1982; Overton et Rulifson, 2009). Il semblerait néanmoins que certains individus résident durant toute leur vie dans les estuaires ou bien fassent des aller-retour entre les milieux d'eaux douces et estuariens (Jessop et al., 2012).

Après le stade de la civelle pigmentée vient celui de l'anguille jaune. Bien que peu différents en terme de pigmentation, ces deux stades se distinguent par leur taille. L'anguille jaune a généralement dépassé 30 cm de longueur et est considérée comme ayant achevé sa phase de migration pour laisser place à une phase de croissance (Martin, 1995). C'est à ce stade qu'intervient la différenciation sexuelle. Contrairement aux premières hypothèses émises dans la littérature, elle ne serait pas liée à l'influence de la latitude (Oliveira et al., 2001). Des théories plus récentes estiment que la différenciation sexuelle pourrait être davantage influencée par la densité des individus en un lieu donné et le type d'habitat concerné : les habitats de type fluviaux seraient caractérisés par de fortes densités d'individus majoritairement mâles tandis que les milieux lacustres seraient davantage occupés par des individus femelles, à des densités moindres (Davey et Jellyman, 2005; Krueger et Oliveira, 1999; Oliveira et al., 2001). Cela expliquerait pourquoi le bassin hydrographique du Saint-Laurent, et plus particulièrement le lac Ontario, est occupé à plus de 95% par des femelles de grande taille qui représenteraient l'essentiel du stock de génitrices de l'espèce américaine (Castonguay et al., 1994; Dutil et al., 1985).

Il peut parfois s'écouler jusqu'à 30 ans (de 8 à 20 ans en moyenne, selon le site) avant que les anguilles d'Amérique acquièrent leur maturité sexuelle (Casselman, 2003), elles atteignent alors le stade d'anguille argentée. En plus de la maturation gonadique, ce stade s'accompagne de modifications morphologiques importantes et nécessaires à la migration en eaux profondes océaniques. On observe entre autres une augmentation de la taille des yeux, une

diminution du nombre de cônes couplée à une augmentation du nombre de bâtonnets de la rétine (augmentation globale de la photosensibilité), une résorption du système digestif et enfin un allongement des nageoires pectorales (Bowmaker et al., 2008; Sorensen et Pankhurst, 1988; Tesch et Henderson, 1977). Il semblerait que les anguilles argentées femelles entament leur migration vers la mer des Sargasses lorsqu'elles atteignent une taille optimale augmentant avec la distance du site de ponte (Jessop, 2010; Tremblay, 2009). Les anguilles argentées cessent de s'alimenter en cours de migration et utilisent la quasi-totalité de leurs réserves lipidiques pour la migration et la maturation. À maturité, ces réserves représentent en moyenne 20% du poids de l'animal chez *A. rostrata* (Tremblay, 2009).

Évidences, causes du déclin et problématique : les contaminants chimiques ont-ils joué un rôle dans la baisse du recrutement de l'espèce ?

A. rostrata a connu un déclin très prononcé de son recrutement au milieu des années 1980. Cela s'est traduit par une diminution drastique (déclin exponentiel estimé à 90% par an) du nombre de juvéniles remontant le fleuve Saint-Laurent en direction du lac Ontario (Castonguay et al., 1994; Haro et al., 2000). Le recrutement pour le système Saint-Laurent n'est jamais revenu à ses valeurs initiales et reste très faible aujourd'hui encore : on estime que le recrutement de l'espèce au cours des années 2000 ne représente que 2% du recrutement tel qu'il était mesuré durant les années 1980 (DFO, 2010) (figure 3). Un plan de gestion à long terme vise à restaurer l'abondance de l'anguille d'Amérique à ses niveaux des années 1980, avec plus ou moins de succès pour le moment selon la zone géographique considérée (DFO, 2010). L'anguille d'Amérique a été désignée comme « espèce préoccupante » par le Comité sur la situation des espèces en péril au Canada en avril 2006 et la réévaluation de son statut vers un statut d'espèce menacée a été entérinée en mai 2012 (COSEPAC, 2006, 2012). L'anguille a un statut d'espèce en voie de disparition en Ontario depuis 2007 et la pêche est interdite dans cette province depuis 2004. Alors que le déclin est très marqué en Ontario et au Québec, situés à la limite septentrionale de l'aire de répartition de l'espèce, l'anguille

d'Amérique est encore abondante au centre de sa distribution (DFO, 2010). Néanmoins, ce sont justement les anguilles femelles situées au nord de l'aire de répartition de l'espèce, à la limite septentrionale, qui possèdent la plus forte fécondité absolue (Tremblay, 2009), ce qui soulève la question de l'impact de leur raréfaction sur le recrutement de l'ensemble de la population.

Quatre causes potentiellement responsables de ce déclin ont été recensées : des changements océaniques globaux (climats, courants), une surexploitation par les pêcheries, des modifications physiques d'origine anthropique de l'habitat de l'anguille (barrages hydroélectriques, espèces invasives) et enfin les contaminants chimiques (Castonguay et al., 1994; COSEPAC, 2006). Néanmoins, aucune de ces causes n'a pu être clairement identifiée comme unique responsable du déclin et c'est probablement une synergie de ces quatre phénomènes qui est responsable de la raréfaction de l'espèce. Des facteurs tels que les variations de l'oscillation nord-atlantique, un réchauffement des eaux de surface de la mer des Sargasses ou encore une diminution de l'intensité du courant du Gulf Stream peuvent théoriquement altérer la survie et la capacité des leptocéphales à rejoindre les côtes nord-américaines (Bonhommeau et al., 2008; Friedland et al., 2007; Knights, 2003; Wirth et Bernatchez, 2003). Toutefois, une influence de ces facteurs climatiques sur le déclin de l'anguille reste à l'heure actuelle hypothétique. Les barrages hydroélectriques semblent cependant être une source importante de mortalité pour les anguilles argentées provenant du lac Ontario, causant 40% de pertes durant la dévalaison (Carr et Whoriskey, 2008; Verreault et Dumont, 2003).

L'impact des pêcheries commerciales sur les stocks d'anguilles du système Saint-Laurent a également pu contribuer au déclin de l'espèce : de Lafontaine et al. (2010) ont modélisé et mis en évidence une diminution des captures par unité d'effort (CPUE) suggérant une surexploitation commerciale de l'espèce entre le milieu des années 1960 et 2000. En 1996 et 1997, le taux de mortalité des anguilles argentées du système Saint-Laurent causé par la pêche commerciale a été estimé à environ 22% (Caron et al., 2003; Verreault et Dumont,

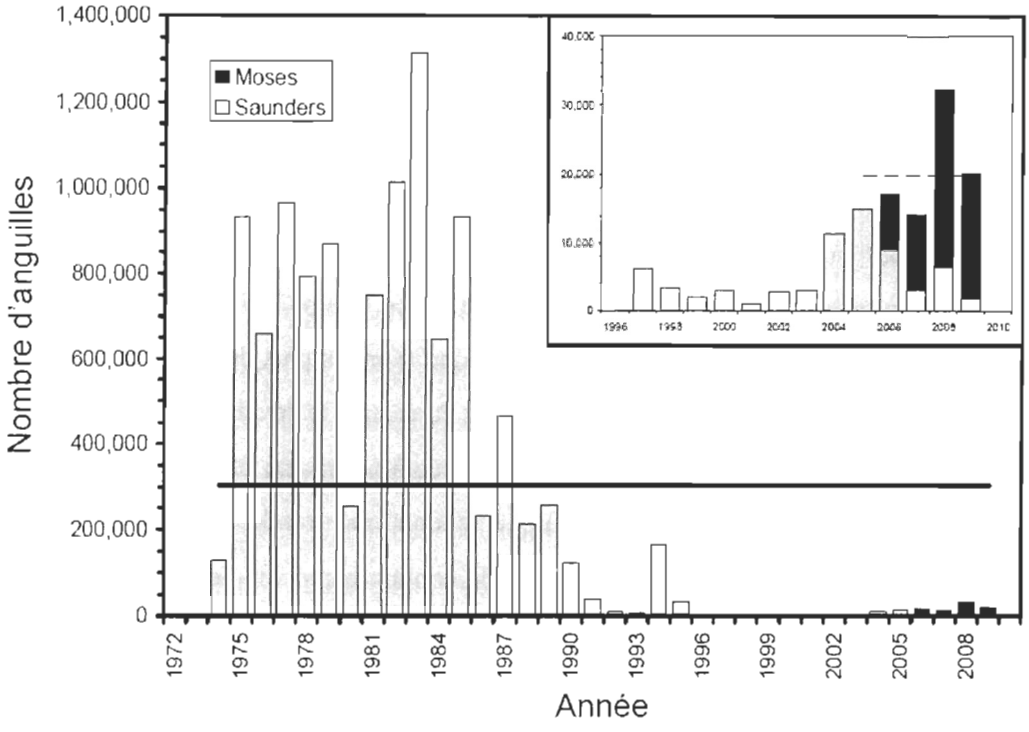

Figure 3: Comptages annuels du nombre d'anguilles remontant aux barrages de Saunders (ON, Canada) et de Moses (NY, USA) en direction du lac Ontario. La station de comptage de Moses a été mise en place en 2006. La ligne pleine représente le recrutement moyen sur toute la série temporelle. La ligne pointillée dans l'encart représente le recrutement moyen sur les cinq dernières années de la série temporelle. Adapté de DFO (2010).

2003). Aujourd'hui, la majorité des pêcheries d'anguilles argentées dans l'estuaire du Saint-Laurent sont fermées et il reste un peu de pêche commerciale à l'anguille dans les secteurs du lac Saint-Pierre, par exemple (COSEPAC, 2012; de Lafontaine et al., 2010). En Ontario, la pêche, la détention, la vente, l'échange ou encore le transport de l'espèce sont prohibés depuis 2004. Au lac St-Pierre (QC), le nombre de permis de pêche est passé de 36 à 6 entre 2002 et 2007. Ces interdictions ou réductions sont le résultat d'un plan de gestion intégré dont l'objectif à court terme est de réduire de 50% toute source de mortalité de l'anguille afin de faire redescendre celle-ci à ses niveaux de la fin des années 1990. Une partie des permis de

pêche rachetés l'ont été par les compagnies exploitant les barrages hydroélectriques en vue de compenser les mortalités causées par ceux-ci (DFO, 2010).

Une autre hypothèse suggère un rôle des contaminants chimiques d'origine anthropique dans le déclin des populations d'anguille d'Amérique et d'Europe (Castonguay et al., 1994; COSEPAC, 2006; Couillard, 2009; Geeraerts et Belpaire, 2010; Robinet et Feunteun, 2002). Comme énoncé précédemment, le cycle vital particulier de l'anguille lui impose d'accumuler de grandes quantités d'énergie sous forme lipidique (parfois plus de 20% du poids total de l'animal) pour subvenir à ses besoins migratoires et assurer le développement gonadique (Boëtius et Boëtius, 1980). De plus, l'espèce est placée à un niveau élevé de la chaîne alimentaire, est soumise à un mode de vie benthique pendant une grande partie de sa vie, ne se reproduit qu'une fois (pas de transferts réguliers de contaminants vers les œufs) et possède une grande longévité. Ces cinq facteurs la rendent susceptible à accumuler de grandes quantités de polluants organiques persistants (POP) lipophiles (Hodson et al., 1992, 1994; Roosens et al., 2008). Ainsi, l'anguille est une des espèces de poissons les plus contaminées (mercure, POP, etc.) du Saint-Laurent (Couillard, 2009; Hodson et al., 1992, 1994). Parmi les composés accumulés par l'anguille, on peut citer par exemple les biphényles polychlorés (BPC), les polychlorodibenzo-*p*-dioxines (PCDD), les polychlorodibenzofuranes (PCDF), les pesticides organochlorés ou encore les diphényléthers polybromés (PBDE). Des concentrations relativement élevées de POP (PCDD, PCDF, BPC, PBDE et pesticides) ont été mesurées chez des anguilles du lac Ontario capturées en 2008, comparativement à d'autres sites au Canada (Byer et al., 2013a,b). Ces concentrations sont toutefois moindres par rapport à celles rapportées historiquement chez des anguilles migratrices capturées dans le Saint-Laurent (Castonguay et al., 1989; Hodson et al., 1994). Avant 2000, les concentrations en composés apparentés aux dioxines (PCDD, PCDF et BPC coplanaires) chez les anguilles d'Amérique ont dépassé les seuils de toxicité décrits chez le touladi du lac Ontario (*Salvelinus namaycush*), suggérant un possible effet sur la qualité des œufs et des génitrices (Byer, 2013). Tous ces contaminants peuvent affecter l'anguille à différents stades de vie et avoir des impacts sur sa croissance, sa reproduction et sa survie (COSEPAC, 2006; Geeraerts et Belpaire, 2010; Robinet et Feun-

teun, 2002). Les stades embryonnaires et larvaires des téléostéens sont parmi les stades de vie les plus sensibles aux contaminants environnementaux (Von Westernhagen, 1988).

Au cours de la maturation sexuelle des anguilles argentées femelles, les réserves lipidiques stockées dans les muscles ainsi que les POP qui y sont associés sont incorporés aux oocytes, et donc aux futurs embryons, à des concentrations supérieures à celles mesurées dans d'autres tissus (Hodson et al., 1992, 1994; Palstra et al., 2007). En effet, au fur et à mesure que les anguilles métabolisent leurs réserves lipidiques durant la migration qui précède la reproduction, la concentration des POP augmente de près de 40% dans les réserves lipidiques résiduelles (Palstra et al., 2006) qui serviront à la production des œufs. Ces contaminants sont susceptibles d'altérer le développement embryonnaire de l'espèce, mais pas seulement, puisque les géniteurs peuvent aussi être affectés (Couillard, 2009; Robinet et Feunteun, 2002; Geeraerts et Belpaire, 2010) (figure 4).

Les stades juvéniles des poissons sont particulièrement sensibles aux composés apparentés aux dioxines (PCDD, polychlorodibenzofuranes ou PCDF, et BPC structurellement proches de ces derniers) qui ont été massivement rejetés dans le lac Ontario, lieu de croissance pour beaucoup de femelles de l'anguille d'Amérique (Allan et Ball, 1990; Tillitt et al., 2008). La quasi-disparition de la population de touladi (*Salvelinus namaycush*) du lac Ontario a été partiellement attribuée à de fortes concentrations de ces composés transférés dans les œufs au cours des années 1960 et 1970, et causant des mortalités embryolarvaires chez cette espèce particulièrement sensible (Cook et al., 2003; Tillitt et al., 2008). Les femelles anguilles résidant parfois jusqu'à une vingtaine d'années au niveau du lac Ontario, il est possible qu'une pollution importante au cours des années 1960 ne montre ses effets sur le recrutement de juvéniles dans le lac Ontario que plusieurs décennies plus tard. Au cours des dernières décennies, les niveaux de contamination des Grands Lacs pour ces composés ont diminué (Bhavsar et al., 2008a; Byer, 2013; Huestis et al., 1996, 1997) et pourraient être responsables d'effets sous-létaux plus subtils. Il est encore difficile d'obtenir des jeunes stades de vie d'anguille en captivité (Oliveira et Hable, 2010), rendant presque impossible l'étude

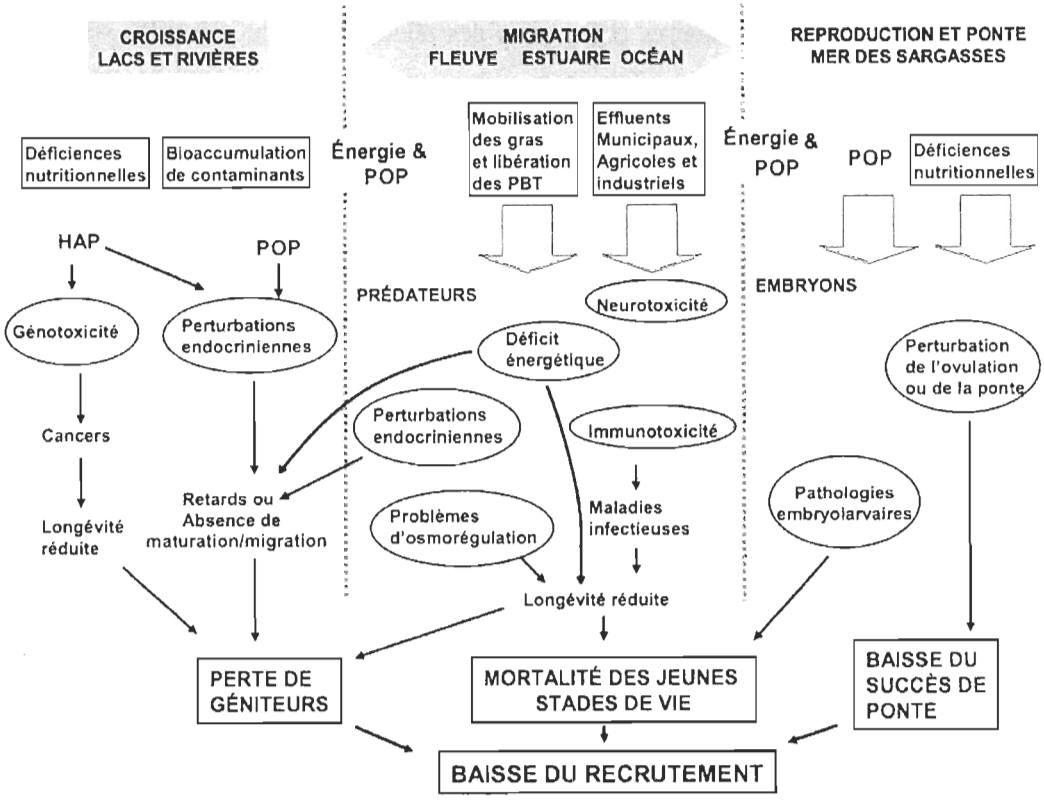

Figure 4: Modèle présentant différentes hypothèses sur le rôle possible des contaminants dans la baisse de recrutement observée chez les anguilles d'Amérique de l'estuaire du Saint-Laurent, à différents stades de leur cycle de vie. Adapté de Couillard (2009).

de l'impact des contaminants sur sa reproduction ou son développement embryonnaire (Geeraerts et Belpaire, 2010). Seuls des travaux relativement récents réalisés sur des embryons de l'espèce européenne suggèrent que les concentrations actuelles en composés apparentés aux dioxines seraient suffisantes pour perturber son développement (Palstra et al., 2006) et soutiennent l'hypothèse émise précédemment dans le cas de l'anguille d'Amérique. Néanmoins, les résultats des travaux de Palstra et al. (2006) doivent être considérés avec précaution étant donné les mortalités embryonnaires élevées mesurées avec les anguilles faiblement contaminées dans l'étude, ainsi que l'utilisation d'un test *in vitro* impliquant des cellules de rat

pour évaluer les quantités de composés apparentés aux dioxines présentes dans les gonades des femelles.

Polluants organiques persistants bioaccumulés par l'anguille d'Amérique

Les polluants organiques persistants sont des composés organiques qui, à des degrés variables, résistent aux processus de dégradations chimiques et biologiques. Leurs propriétés hydrophobes et lipophiles les rendent susceptibles d'être bioaccumulés au sein de la chaîne alimentaire. Cette section traite de quelques-uns des principaux groupes de POP retrouvés dans les tissus des anguilles d'Amérique dont il a été question précédemment (PCDD, PCDF, BPC, etc.) et dont il sera question plus tard dans le présent manuscrit, avec une emphase sur leurs effets embryotoxiques et neurotoxiques chez les jeunes stades des poissons.

Les dioxines et composés apparentés

Généralités

Le terme dioxine est un terme général qui désigne plusieurs composés similaires du point de vue de leur structure chimique (Mandal, 2005), mais il désigne plus spécifiquement les polychlorodibenzo-*p*-dioxines (PCDD). Leur structure chimique (figure 5) comprend deux cycles benzènes liés par deux atomes oxygène (O). Chacun des deux cycles peut porter jusqu'à 4 atomes de chlore (Cl), permettant un total de 75 combinaisons uniques communément appelées congénères. On inclut aussi sous le terme de « composés apparentés aux dioxines », ou encore composés coplanaires, les polychlorodibenzofuranes (PCDF) et certains biphényles polychlorés (BPC). Les PCDF, dont on dénombre 135 congénères différents, sont structurellement très proches des PCDD, mais ne possèdent qu'un seul atome d'oxygène entre les deux cycles benzènes (figure 5). Les BPC sont constitués de deux cycles benzènes liés par une liaison covalente carbone-carbone (figure 5). Seuls un petit nombre des 209 congénères

possibles de BPC sont apparentés aux dioxines. Parmi eux, les BPC non-ortho-substitués (qui ne possèdent pas d'atomes de chlore aux positions 2, 6, 2' et 6') sont au nombre de 4 (BPC77, BPC81, BPC126 et BPC169) et sont également appelés BPC coplanaires. Ce sont les BPC les plus toxiques, car structurellement plus proches des PCDD. On inclut également parmi les composés apparentés aux dioxines les BPC mono-ortho-substitués, au nombre de 8, qui eux possèdent un atome de chlore en position 2. Ils sont néanmoins réputés moins toxiques que les BPC coplanaires, notamment chez les poissons (Van den Berg et al., 1998).

Les PCDD et PCDF sont des sous-produits non désirés introduits accidentellement dans l'environnement et issus principalement de processus de combustion (Hites, 2010). Identifiées au cours des années 1970 et 1980, les principales sources de contamination de l'environnement par ces composés incluent les incinérateurs municipaux, le traitement du bois au pentachlorophénol, le raffinage du pétrole, l'industrie des pâtes et papiers ou encore l'utilisation massive de certains herbicides tels que l'Agent Orange (Kutz et al., 1990; Pirkle et al., 1989). Parmi les PCDD et PCDF, le composé le plus connu est sans doute la 2,3,7,8-tétrachlorodibenzo-*p*-dioxine, communément appelée TCDD. Elle est considérée comme le composé le plus toxique parmi tous les PCDD et autres composés apparentés aux dioxines, et est probablement le plus étudié d'entre eux. Les BPC, en revanche, ont été produits et commercialisés volontairement, principalement par Monsanto (États-Unis et Angleterre), Bayer (Allemagne), Prodelec (France), Kanegafuchi (Japon) ou encore Caifaro (Italie), en raison de leur stabilité chimique, résistance au feu et propriétés diélectriques offrant une vaste gamme d'usages à l'industrie : les BPC ont notamment été utilisés comme fluides thermiques, lubrifiants hydrauliques ou isolants électriques. La production et l'utilisation des BPC à l'échelle mondiale couplées à des pratiques d'élimination inadéquates ont conduit à leur introduction massive dans l'environnement (Safe, 1993). Les PCDD, PCDF et BPC sont tous lipophiles et classés comme polluants organiques persistants (POP), car ils sont résistants à la dégradation aussi bien chimique que biologique et sont bioaccumulés au sein de la chaîne alimentaire (Hutzinger et al., 1985; Tanabe, 1988). Cela concerne tout particulièrement le milieu aquatique, le sédiment jouant le rôle de puits pour l'ensemble des POP (Zoumis et al., 2001). En

plus de la voie trophique, les organismes benthiques sont particulièrement exposés dans la mesure ou les POP peuvent être relargués dans la colonne d'eau (Booij et al., 1992). En raison de leur omniprésence dans l'environnement et de l'inquiétude croissante relative à leurs possibles effets sur la santé humaine, la production, l'importation et l'usage des BPC ont été restreints au cours des années 1970 avant d'être interdits en 1977 en Amérique du Nord (Ross, 2004). En Europe, il faut attendre 1998 et la convention d'Aarhus pour qu'un accord visant à bannir l'usage et la production de BPC soit signé entre les 39 pays concernés (Jones et de Voogt, 1999). Malgré ces réglementations, les BPC accumulés dans les sédiments et les tissus des organismes aquatiques deumerent une source de contamination active (van der Oost et al., 1996).

Mécanisme de toxicité

Les réponses biologiques des dioxines et composés apparentés aux dioxines sont modulées par leur liaison à un récepteur protéique cellulaire, le récepteur Ah (aryle hydrocarbure) (Poland et Glover, 1976). Il est hautement conservé chez les vertébrés (Hahn, 2002; Hahn et Karchner, 1995) et fait partie de la famille des protéines hélice-boucle-hélice : il s'agit d'un récepteur possédant de nombreux agonistes endogènes et ayant un rôle important dans le développement embryonnaire (Nguyen et Bradfield, 2007). Les composés apparentés aux dioxines susceptibles de se lier au récepteur Ah ont une meilleure affinité pour celui-ci lorsque leurs atomes de chlore sont situés en position latérale, c'est-à-dire lorsque leur squelette chimique se rapproche de celui de la TCDD (Safe, 1990). Plus généralement, cette affinité dépend du degré de chloration et de la disposition des atomes de chlore sur le cycle aromatique. Les différentes espèces de poissons présentent une grande variabilité du nombre, type et patron d'expression des gènes liés au récepteur Ah (Zhou et al., 2010). Certaines populations de poissons ont développé une résistance aux dioxines qui serait liée au polymorphisme du gène codant pour le récepteur Ah. C'est le cas, par exemple, du choquemort (*Fundulus heteroclitus*) (Proestou et al., 2014; Reitzel et al., 2014). Plus généralement, les

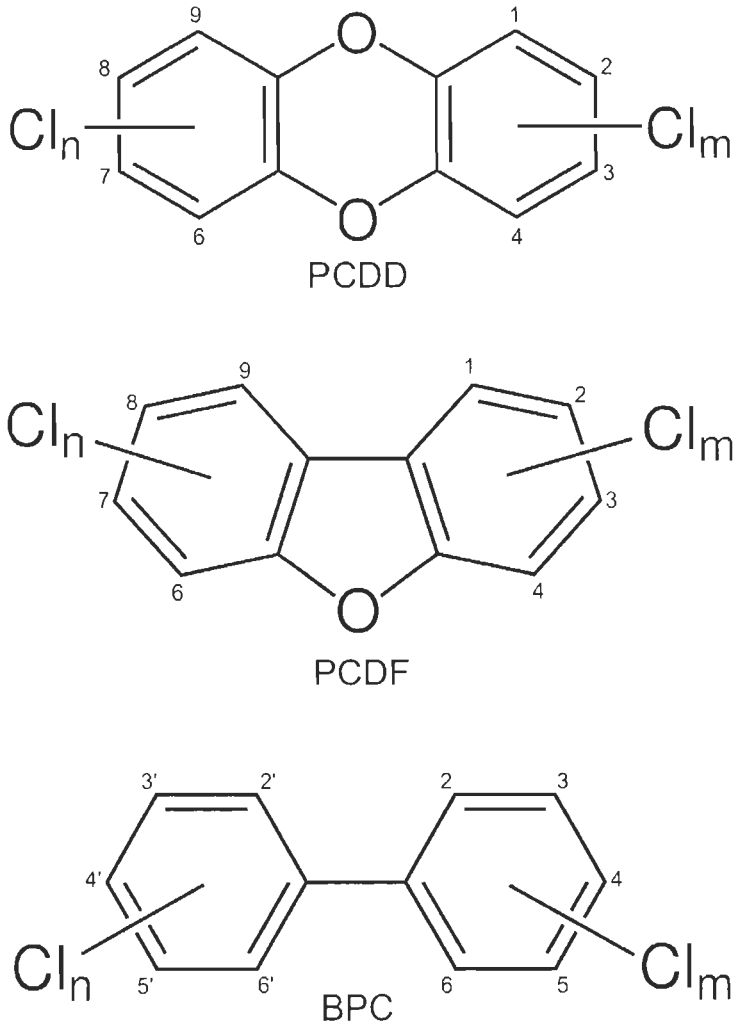

Figure 5: Structure générale et positions de substitution des atomes de chlore pour les PCDD, PCDF et BPC.

poissons possèdent une large variété de clades du récepteur Ah (AhR1, AhR2 et AhR3), possédant eux mêmes plusieurs isoformes (Doering et al., 2013). Chez certaines espèces de poissons, il a été démontré que les composés apparentés aux dioxines ne sont pas capables de se lier au récepteur AhR1, et que c'est AhR2 qui régule la toxicité de ces derniers (Antkiewicz et al., 2006; Clark et al., 2010). Néanmoins, certaines isoformes de AhR1 semblent jouer un rôle dans le développement embryonnaire et/ou la régulation de la toxicité des composés apparentés aux dioxines, comme cela a été récemment mis en évidence chez le poisson zèbre (*Danio rerio*) (Garner et al., 2013).

Dans l'environnement, les PCDD, PCDF et BPC sont retrouvés sous la forme de mélanges complexes constitués de nombreux congénères et dont la composition relative diffère d'un niveau trophique à l'autre. Ces différences sont causées par la dégradation environnementale plus ou moins rapide de certains congénères, ou en raison de différences en termes de solubilité ou volatilité entre ces derniers. La composition de ces mélanges varie dans le temps et l'espace, et ne ressemble que très peu à celle des mélanges commerciaux (par exemple, l'Aroclor, dans le cas des BPC) originellement produits par l'industrie et introduits dans l'environnement (Van den Berg et al., 1998). La toxicité de mélanges complexes de composés apparentés aux dioxines peut être prédite sur la base de leur concentration en EQT-TCDD (équivalent toxique TCDD) totale, soit la somme de la concentration de chaque composé multiplié par son FET (facteur d'équivalence toxique) propre. Le FET d'un composé représente la toxicité relative de celui-ci vis-à-vis de la TCDD, dont il est admis qu'elle est la dioxine la plus toxique (son FET est égal à 1) et qui sert donc de référence (Van den Berg et al., 1998; Walker et Peterson, 1991; Zabel et al., 1995b). Ce modèle théorique requiert que chacun des composés apparentés aux dioxines agisse selon le même mécanisme, produise les mêmes effets, et que ces effets soient additifs dans le cas d'un mélange de ces composés. Certaines études chez les salmonidés semblent toutefois montrer que les composés apparentés aux dioxines penta-substitués (par exemple, le 2,3,4,7,8-pentachlorodibenzofurane) pourraient être plus nocifs que les tétra-substitués, dont la TCDD (Parrott et al., 1995). Les FET semblent varier d'une espèce à l'autre, y compris chez les poissons (Chen et Cooper,

1999). De plus, la sensibilité des différentes espèces de poissons à la TCDD varie également selon l'espèce étudiée, le stade de vie considéré et selon la réponse évaluée (Elonen et al., 1998). Parmi les poissons, les salmonidés figurent parmi les plus sensibles aux dioxines et composés apparentés (Elonen et al., 1998; Walker et al., 1991), ce qui pourrait en partie expliquer le déclin et l'échec de la restauration du touladi dans le lac Ontario (Cook et al., 2003; Tillitt et al., 2008) évoqué précédemment. À titre de comparaison, le poisson zèbre (*Danio rerio*), l'une des espèces les plus résistantes connues, est environ 40 fois moins sensible que le touladi (Doering et al., 2013; Elonen et al., 1998). Ces différences interspécifiques en termes de sensibilité à la TCDD pourraient entre autres être liées aux propriétés structurelles du récepteur Ah, tel que cela a déjà été démontré chez les oiseaux (Doering et al., 2013). Deux composés apparentés aux dioxines peuvent partager un mécanisme d'action commun (la liaison au récepteur Ah) mais induire des réponses sensiblement différentes via différentes voies d'effets telles que des interactions croisées impliquant le translocon nucléaire du récepteur aryle hydrocarbure (ARNT) (Olufsen et Arukwe, 2011), la production de dérivés réactifs de l'oxygène (DRO) (Arzuaga et al., 2006) ou encore des voies de signalisation (Gjernes et al., 2012) pouvant altérer la réponse de l'organisme.

Malgré cinq décennies de recherche intensive, les mécanismes de toxicité de la TCDD et des composés apparentés ne sont pas encore complètement compris (King-Heiden et al., 2012). Seules les premières étapes sont relativement bien connues (figure 6). Lorsqu'ils circulent librement dans l'organisme, les composés apparentés aux dioxines peuvent se lier aux récepteurs Ah localisés dans le cytoplasme. Ainsi activé, le récepteur Ah subit alors une translocation dans le noyau cellulaire où il va former un dimère avec un ARNT (translocon nucléaire du récepteur aryle hydrocarbure). Ce complexe dioxine/AhR/ARNT peut se lier aux séquences spécifiques (nommées AhRE, pour élément de réponse au récepteur Ah) des promoteurs d'une batterie de gènes et modifier leur expression (Hahn et Hestermann, 2008; Mandal, 2005). Le plus connu et étudié d'entre eux est certainement le gène du cytochrome P4501A1 (*cyp1a*) qui code pour l'enzyme de détoxification cytochrome P450 (Withlock, 1999). En plus de modifier l'expression de certains de leurs gènes cibles, les composés

apparentés aux dioxines sont également des compétiteurs exogènes vis-à-vis des molécules endogènes capables de se lier au récepteur Ah. Ils peuvent induire des perturbations en empêchant l'activation programmée et l'expression de certains gènes.

Si le rôle du récepteur Ah dans les mécanismes de toxicité des dioxines est clairement établi chez les mammifères (Fernandez-Salguero et al., 1996) et les poissons téléostéens (Antkiewicz et al., 2006; Prasch et al., 2003), celui du cytochrome P450 (CYP1A) est plus controversé. À ce jour deux travaux contradictoires réalisés chez le poisson zèbre (*Danio rerio*) ont été publiés. Le premier statue sur un mécanisme d'embryotoxicité de la TCDD CYP1A-dépendant (Teraoka et al., 2003) tandis que le second conclut, à l'inverse, sur un mécanisme indépendant du CYP1A (Carney et al., 2004). Les deux groupes d'auteurs ont utilisé le même outil, zfCYP1A-MO, un morpholino [2] spécifique du gène zfCYP1A chez le poisson zèbre. Une étude plus récente tend à confirmer que le blocage de l'expression du cytochrome P450 ne supprime pas les effets embryotoxiques de la TCDD observés chez le poisson zèbre (Antkiewicz et al., 2006).

L'implication du CYP1A dans la production de dérivés réactifs de l'oxygène (DRO) est en revanche bien connue et responsable de l'apparition de stress oxydant ainsi que de l'induction d'apoptose (Nebert et al., 2000; Schlezinger et al., 2006). Ces effets ont pu être mis en évidence chez différents poissons modèles exposés à la TCDD (Cantrell et al., 1998, 1996; Dong et al., 2001; Toomey et al., 2001) et bloqués par l'utilisation d'antioxydants ou d'inhibiteurs du cytochrome P450 (Dong et al., 2002, 2004). L'induction du CYP1A est susceptible d'induire l'apparition de DRO en oxydant des composés aussi bien endogènes qu'exogènes. Le stress oxydant peut déclencher la mort cellulaire via plusieurs voies, incluant les dommages à l'ADN, les perturbations des flux ioniques, du volume cellulaire ou encore du pH intracellulaire. Il existe des processus de contrôle de ce stress oxydant, par l'activation de gènes

2. Désigne une molécule synthétique qui se fixe aux séquences complémentaires des ARN par appariement de bases et qui est employée pour intervenir dans le processus de l'expression génétique. Ainsi, les oligonucléotides morpholinos sont utilisés pour empêcher certaines molécules d'accéder aux séquences spécifiques de molécules d'acide nucléique.

spécifiques non P450 via l'EPRE (éléments de réponses électrophiles) ou par l'intermédiaire de ligands endogènes ou exogènes du récepteur Ah. Le récepteur Ah est donc impliqué dans un système complexe de régulation tant positive que négative du stress oxydant au niveau cellulaire (Nebert et al., 2000) (figure 6). Ainsi, le rôle du cytochrome P450 reste encore à préciser, bien que certains auteurs l'excluent d'ores et déjà de leurs schémas mécanistiques (Carney et al., 2006; Tillitt et al., 2008).

D'autres pistes que celles impliquant le CYP1A sont étudiées pour comprendre le mécanisme d'action des dioxines. Des travaux récents chez le poisson zèbre (Teraoka et al., 2009, 2014) suggèrent que COX2 (cyclo-oxygénase 2, une isoenzyme impliquée dans la production des prostaglandines) joue un rôle dans la réduction du flux sanguin de la veine mésencéphalique causée par la TCDD (voir ci-après). En effet, des inhibiteurs de COX2 ou le blocage de la transcription du gène codant pour celle-ci ont pour effet d'annuler cet effet de la TCDD. De même, le blocage de la voie du thromboxane (une hormone produite indirectement via les cyclo-oxygénases) par des antagonistes du thromboxane ou son activation par des agonistes de celui-ci annulent ou miment (respectivement) cet effet de la TCDD. Les résultats de ces auteurs (Teraoka et al., 2009, 2014) suggèrent l'existence d'une nouvelle voie impliquant COX2 ainsi que le thromboxane dans les mécanismes d'embryotoxicité de la TCDD chez les poissons.

Embryotoxicité et neurotoxicité des composés apparentés aux dioxines

Les signes d'embryotoxicité causés par les composés apparentés aux dioxines sont relativement bien connus et ont été décrits chez de nombreuses espèces de poissons, en particulier le poisson zèbre. Le tableau 1 dresse une liste des études récentes (de 2000 à aujourd'hui) en rapport avec les effets embryotoxiques et neurotoxiques des composés apparentés aux dioxines chez les jeunes stades de vie (embryons et larves) des poissons. D'une manière générale, aucun signe de toxicité apparente n'est observé lors des premières phases de développement précédant l'organogénèse (Tillitt et al., 2008). L'âge de l'appari-

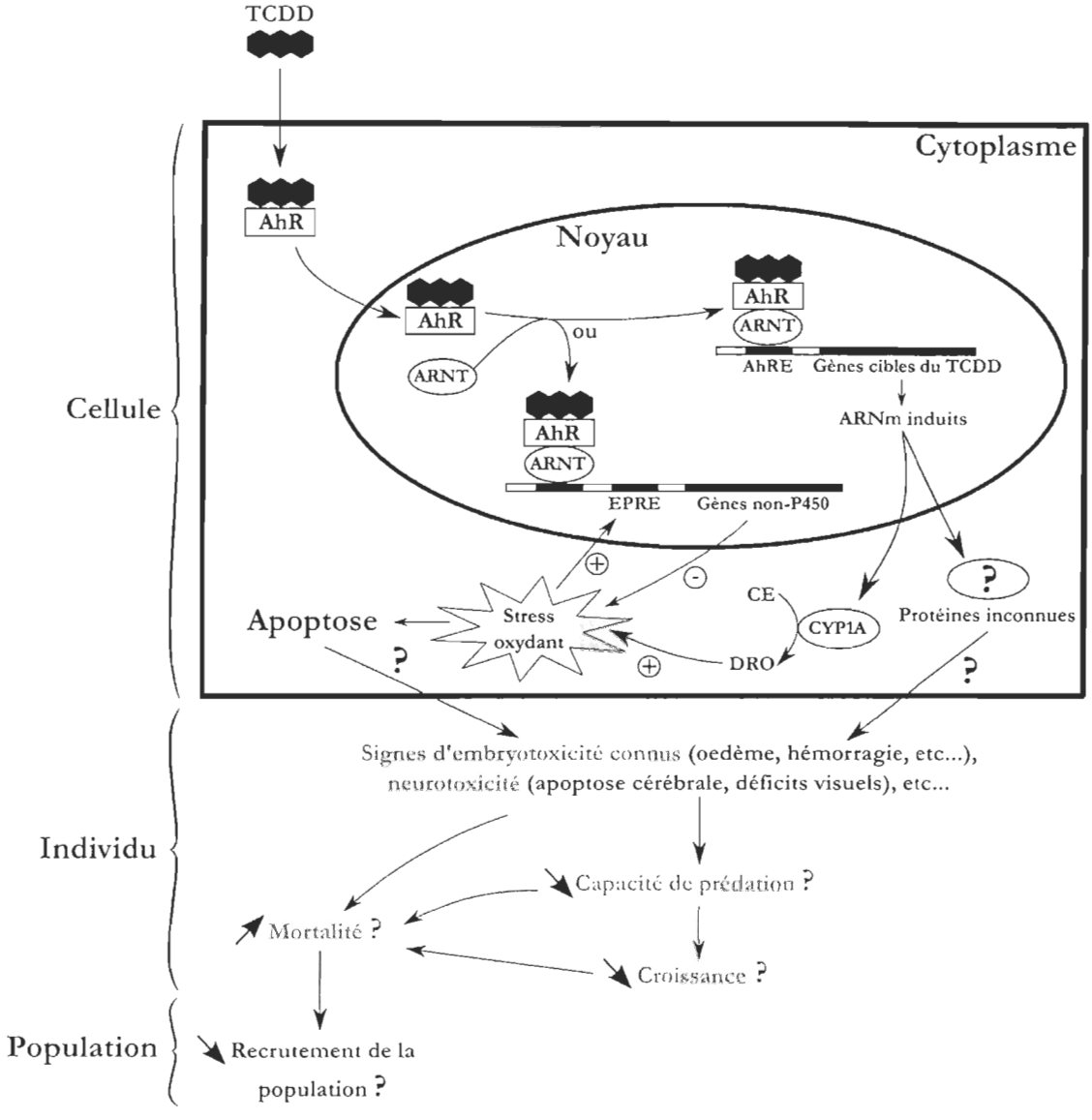

Figure 6: Représentation schématique au niveau cellulaire de la voie de signalisation du récepteur Ah qui régule la toxicité des composés apparentés aux dioxines, ici avec l'exemple de la TCDD (Carney et al., 2006; Nebert et al., 2000), et des effets embryotoxiques et neurotoxiques possibles à l'échelle de l'individu et de la population. CE : composés endogènes ou exogènes ; DRO : dérivés réactifs de l'oxygène ; EPRE : éléments de réponses électrophiles.

tion des premières lésions varie selon l'espèce considérée, et n'est pas forcément lié au temps nécessaire à son développement. Les salmonidés possèdent un temps de développement relativement long et sont parmi les poissons les plus sensibles à la TCDD, tandis que le poisson zèbre figure parmi les espèces les plus résistantes et possède un temps de développement très court (l'éclosion intervient après 48-72 heures post-fécondation). En revanche, le tête-de-boule (*Pimephales promelas*), qui fait partie de la même famille que le poisson zèbre et qui possède des stades de développement rapides et très proches de ce dernier, est considéré comme à peine moins sensible à la TCDD que les salmonidés (Elonen et al., 1998).

Hormis un retard de croissance, la réponse embryotoxique aux dioxines la plus fréquente rencontrée chez les poissons est l'œdème péricardiaque et/ou du sac vitellin, causé par une perturbation des fonctions circulatoires et osmorégulatrices, des malformations cardiovasculaires ou encore une augmentation de la perméabilité vasculaire, également responsable d'hémorragies (Belair et al., 2001; Henry et al., 1997; Hill et al., 2004a,b; Teraoka et al., 2002; Walker et al., 1991). Les composés apparentés aux dioxines affectent également la chondrogenèse dans son ensemble, cela se traduisant par une atrophie parfois prononcée de la mâchoire inférieure, bien que la croissance de l'ensemble du cartilage crânien soit altérée (Hill et al., 2004a; Hornung et al., 1999; Kim et Cooper, 1999; Teraoka et al., 2006). L'ensemble de ces réponses est typique du syndrome de la maladie du sac bleu (MSB, de l'anglais « *blue sac disease* ») (Spitsbergen et al., 1991) et dépendant de la liaison des composés coplanaires avec le récepteur Ah (Prasch et al., 2003).

Les réponses embryotoxiques citées jusqu'ici (œdèmes et malformations cardiaques, notamment) sont des réponses dites sévères qui entraînent généralement la mort de l'embryon ou de la larve concernée, comme cela a été le cas dans le rôle possible des dioxines dans le déclin du touladi dans le lac Ontario lors des années 60-70 (Cook et al., 2003; Tillitt et al., 2008; Wright et Tillitt, 1999). Hormis dans le cas d'espèces extrêmement sensibles telles que le touladi, la mise en évidence d'effets aussi sévères requiert l'usage de doses d'exposition souvent peu réalistes d'un point de vue environnemental. À des doses plus réalistes,

Tableau 1: Études récentes (de 2000 à 2014) en rapport avec les effets embryotoxiques et neurotoxiques des composés apparentés aux dioxines chez les jeunes stades de vie (embryons et larves) des poissons.

Composé	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Référence
TCDD	Syndrome MSB[a], Dysfonction hémodynamique	Touladi (*Salvelinus namaycush*)	Balnéation (acétone)	3-100 ppt	23-529 pg g^{-1} p.h.[b]	Guiney et al. (2000)
BPC126	Mortalité	Poisson zèbre (*Danio rerio*)	Injection IVi[c] (huile d'arachide)	1 µmol kg^{-1}	n.d.[d]	Westerlund et al. (2000)
TCDD	Syndrome MSB, ↗EROD, Féminisation	*Gobiocypris rarus*	Balnéation (acétone)	2-100.000 pg L^{-1}	n.d.	Wu et al. (2001)
TCDD	Perturbations cardiovasculaires	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO[e])	6 ng L^{-1}	n.d.	Belair et al. (2001)
TCDD	Apoptose mésencéphale	Poisson zèbre (*Danio rerio*)	Balnéation	0.2-1.0 ppb	n.d.	Dong et al. (2001)
TCDD	Syndrome MSB, Mortalité, Apoptose (cerveau, yeux,...), ↗EROD	Choquemort (*Fundulus heteroclitus*)	Injection IVi (trioléine)	25-20.000 pg g^{-1} p.h.	25-20.000 pg g^{-1} p.h.	Toomey et al. (2001)

Tableau I (suite)

Composé	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Référence
TCDD	Apoptose mésencéphale, ↘Afflux sanguin cerveau	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	0.1-1.0 ppb	n.d.	Dong et al. (2002)
TCDD	Malformations osseuses, Caillots sanguins, Dommages vasculaires	Medaka (*Oryzias latipes*)	Balnéation (acétone)	1.55 nM	n.d.	Kawamura et Yamashita (2002)
TCDD	Malformations crânio-faciales, ↘Afflux sanguin	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	0.1-1.0 ppb	n.d.	Teraoka et al. (2002)
BPC126	Malformations cardiaques, ↗EROD	Choquemort (*Fundulus heteroclitus*)	Balnéation (acétone)	5-300 ng L^{-1}	n.d.	Wassenberg et al. (2002)
TCDD	↘Nombre neurones, ↘Expression *neurogenin*	Poisson zèbre (*Danio rerio*)	Balnéation (acétone)	≤ 120 ng L^{-1}	n.d.	Hill et al. (2003)

Tableau I (suite)

Composé	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Référence
TCDD	↘Croissance de la veine cardinale (cœur)	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	0.1-10 ng mL^{-1}	n.d.	Bello et al. (2004)
TCDD	Syndrome MSB, Mortalité	Truite arc-en-ciel (*Oncorhynchus mykiss*)	Injection IVi (trioléine)	38-1000 pg g^{-1} p.h.	38-1000 pg g^{-1} p.h.	Carvalho et al. (2004)
TCDD	↘Nombre celllules ganglionnaires rétine, ↘Vision, ↘Capacité prédation	Truite arc-en-ciel (*Oncorhynchus mykiss*)	Injection IVi (trioléine)	38-300 pg g^{-1} p.h.	38-300 pg g^{-1} p.h.	Carvalho et Tillitt (2004)
TCDD	Malformations crânio-faciales, ↘Croissance larvaire	Poisson zèbre (*Danio rerio*)	Balnéation (acétone)	20-120 ng L^{-1}	n.d.	Hill et al. (2004a)
TCDD	Syndrome MSB	Poisson zèbre (*Danio rerio*)	Balnéation (acétone)	0.1-10 ng L^{-1}	n.d.	Hill et al. (2004b)
TCDD	Malformations cardiaques	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	1 ppb	n.d.	Antkiewicz et al. (2005)

Tableau 1 (suite)

Composé	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Référence
BPC126	↗EROD, ↗DRO^{f}, Stress oxydant	Choquemort (*Fundulus heteroclitus*)	Balnéation (DMSO)	0.003-1.5 nM	n.d.	Arzuaga et al. (2006)
TCDD	Mortalité, Syndrome MSB, Malformations crânio-faciales	Dorade japonaise (*Pagrus major*)	Balnéation (nonane)	3.1-100 µg L^{-1}	60-1550 pg g^{-1} p.h.	Yamauchi et al. (2006)
BPC126	Mortalité	Sole commune (*Solea solea*)	Balnéation (DMSO)	0.1-1000 ng L^{-1}	5.9-33 ng g^{-1} de lipide	Foekema et al. (2008)
BPC126	Malformations crânio-faciales et cardiaques	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	7.5 µg L^{-1}	n.d.	Grimes et al. (2008)
TCDD	Malformations des valves cardiaques	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	1 ng mL^{-1}	n.d.	Mehta et al. (2008)
TCDD	Mortalité, Syndrome MSB, ↗CYP1A mRNA	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	50 pg mL^{-1}	n.d.	Wu et al. (2008)
TCDD	Malformations crânio-faciales	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	0.01-1.0 ng mL^{-1}	n.d.	Xiong et al. (2008)

Tableau 1 (suite)

Composé	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Référence
BPC126	Syndrome MSB, ↗SOD1 (superoxyde dismutase)	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	100 nM	n.d.	Na et al. (2009)
BPC126	Mortalité, ↗CYP1A	*Paralichthys dentatus*	Balnéation (acétone)	0.0021-2100 ng L^{-1}	0.26-2903 ng g^{-1} p.h.	Soffientino et al. (2010)
TCDD	Malformation des vaisseaux sanguins au cerveau	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	0.3-2.0 ppb	n.d.	Teraoka et al. (2010)
TCDD	Malformation du système nerveux périphérique	Dorade japonaise (*Pagrus major*)	Balnéation (toluène)	3.1-100 µg L^{-1}	n.d.	Iida et al. (2010)
BPC126	↗EROD, ↘Croissance, ↘Activité locomotrice, ↘Capacité prédation	Choquemort (*Fundulus heteroclitus*)	Topique (DMSO)	2.5-50 pg œuf^{-1}	n.d.	Couillard et al. (2011)

Tableau 1 (suite)

Composé	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Référence
BPC77	Syndrome MSB, Perturbation système vasculaire, Développement osseux anormal	Saumon Atlantique (*Salmo salar*)	Balnéation (éthanol)	1-10 ng L^{-1}	n.d.	Olufsen et Arukwe (2011)
TCDD et BPC126	Mortalité, ↘Croissance, ↘Taille tête, ↘Taille yeux	Esturgeon à museau court (*Acipenser brevirostrum*) et Esturgeon noir (*Acipenser oxyrinchus*)	Balnéation (acétone)	0.001-100 ppb (TCDD) et 0.01-1000 ppb (BPC126)	1-30 ng g^{-1} pour 0.1-10 ppb de BPC126	Chambers et al. (2012)
TCDD	Malformations osseuses et cartilagineuses	Medaka (*Oryzias latipes*)	Balnéation (DMSO)	0.01-1.0 ppb	n.d.	Dong et al. (2012)

Tableau 1 (suite)

Composé	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Référence
TCDD	Malformation du système nerveux périphérique, ↘Taille axone du nerf facial	Dorade japonaise (*Pagrus major*)	Balnéation (toluène)	0.1-1.7 µg L^{-1}	n.d.	Iida et al. (2013)
BPC126	Mortalité, Syndrome MSB	Sole commune (*Solea solea*)	Balnéation (DMSO)	0.003-0.03 µg L^{-1}	1400-1700 pg g^{-1} de lipide	Foekema et al. (2014)
TCDD	Mortalité, Malformations crânio-faciales, ↘Capacité prédation	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	25-100 pg mL^{-1}	n.d.	Chollett et al. (2014)

[a] Syndrome de la maladie du sac bleu. Voir texte pour détails.

[b] Poids humide

[c] Intravitelline.

[d] Non déterminé par le ou les auteur(s).

[e] Diméthylsulfoxyde.

[f] Dérivés réactifs de l'oxygène.

Le signe ↗ est utilisé pour signifier une augmentation. Le signe ↘ est utilisé pour signifier une diminution

des réponses plus subtiles telles que des effets sur la croissance, le système immunitaire, le système neuroendocrinien ou la différentiation sexuelle peuvent apparaître (Johnson et al., 2013).

En exposant des embryons de poissons zèbre à des doses équivalentes ou inférieures aux doses minimales requises pour induire l'apparition de signes sévères, Hill et al. (2003) ont mis en évidence divers effets tels qu'une diminution du volume cérébral ainsi qu'une diminution de l'expression de gènes impliqués dans la neurogenèse, *neurogenin* et *sonic hedgehog*. D'autres effets potentiellement neurotoxiques de la TCDD ont été mis en évidence lors du développement embryonnaire chez le poisson zèbre ((Dong et al., 2001), le médaka japonais (*Oryzias latipes*) (Cantrell et al., 1996, 1998) et enfin le choquemort (*Fundulus heteroclitus*) (Toomey et al., 2001) : ces effets se traduisent par une induction de l'activité apoptotique dans le mésencéphale dorsal. Dans le cas du poisson zèbre, Dong et al. (2001, 2002, 2004) ont mis en évidence qu'une diminution du flux sanguin dans la veine mésencéphalique était préalable à l'induction apoptotique dans le mésencéphale, et que ces deux réponses étaient annulées par l'utilisation d'antioxydants, d'inhibiteurs du cytochrome P450 ou de morpholinos dirigés contre le gène codant pour le récepteur Ah.

Les effets neurotoxiques des dioxines sur le comportement des jeunes stades larvaires contaminés est très peu connue. Chez les jeunes stades de poissons, l'altération de paramètres tels que la capacité de prédation, la réponse de fuite ou encore l'activité de base (capacité natatoire) peut avoir des effets significatifs sur le recrutement des populations exposées à des contaminants (Weis et al., 2001, 2003). Peu d'études publiées à ce jour examinent l'effet de composés apparentés aux dioxines sur le comportement de larves de poissons en relation avec des réponses embryotoxiques mesurées de routine (tableau 1). Carvalho et Tillitt (2004) ont exposé des embryons de truite arc-en-ciel (*Oncorhynchus mykiss*) à de la TCDD par injection intravitelline (IVi) et relevé lors des stades larvaires une induction dose-dépendante du cytochrome P450 dans les tissus vasculaires cérébraux et la choroïde, associée à une diminution également dose dépendante du nombre de cellules ganglionnaires de la rétine. Ces

mêmes auteurs ont par ailleurs observé des perturbations des fonctions visuelles (réaction à la lumière et détection de mouvements) ainsi qu'une réduction de la capacité de capture des larves exposées, mais seulement aux plus fortes doses testées (≥300 pg g^{-1}) dans le cas de ce dernier paramètre. Plus récemment, il a été mis en évidence que des doses sous-létales de composés coplanaires, non nécessairement associées au syndrome de MSB, étaient susceptibles d'altérer la capacité de prédation des larves du choquemort et du poisson zèbre (Chollett et al., 2014; Couillard et al., 2011).

D'une manière générale, on remarque que très peu d'études (seulement 6 sur les 35 études récentes listées dans le tableau 1) fournissent les doses internes mesurées dans les tissus des larves ou embryons ou emploient un mode d'exposition qui permet d'avoir une idée précise de la dose interne sans avoir besoin de la mesurer. De même, un grand nombre d'études (30 sur les 35 études répertoriées dans le tableau 1) emploient la balnéation comme méthode pour exposer les embryons. En plus d'une possible adsorption des composés sur les parois des microplaques (Bello et al., 2004), il est reconnu que différents composés ne pénètrent pas dans les mêmes proportions à travers le chorion et que cela dépend de leur coefficient de partage octanol/eau (Helmstetter et Alden III, 1995). Pour toutes les raisons citées précédemment, la plupart des études listées dans le tableau 1 fournissent des résultats qui sont difficiles à comparer avec ce qui se passe réellement sur le terrain, à des doses réalistes d'un point de vue environnemental.

Les BPC non-coplanaires

Outre les BPC coplanaires cités précédemment, la majorité des BPC sont dit non-coplanaires et possèdent au moins deux atomes de chlore aux positions 2, 6, 2' et/ou 6' (figure 5). Ils sont les constituants principaux des mélanges commerciaux tels que l'Aroclor. Leurs mécanismes de toxicité sont indépendants du récepteur Ah pour lequel ils n'ont pas ou peu d'affinité (Kafafi et al., 1993). Les effets toxiques des BPC non-coplanaires ont surtout été étudiés chez les mammifères, très peu chez les oiseaux ou les poissons. L'un des effets

toxiques des BPC non-coplanaires communs aux mammifères, aux poissons et aux oiseaux est la narcose : il s'agit d'un mécanisme de toxicité non spécifique susceptible d'être provoqué chez n'importe quel organisme et par n'importe quel type de composé organique, si l'exposition a lieu a une concentration suffisamment forte (Henry et DeVito, 2003). Chez les mammifères, les BPC non-coplanaires sont bien connus pour induire des effets neurotoxiques. Par exemple, une exposition néonatale de souris à du BPC52 (de 0.7 à 14 μmol kg^{-1}) est susceptible d'induire des changements comportementaux tels qu'une altération des capacités de mémorisation et d'apprentissage au stade adulte (Eriksson et Fredriksson, 1996). Des études *in vitro* chez les mammifères suggèrent que les BPC non-coplanaires sont capables d'altérer le contenu cellulaire et le transport de plusieurs neurotransmetteurs, dont la dopamine, la sérotonine et le glutamate (Mariussen et Fonnum, 2001; Shain et al., 1991). L'un des mécanismes possibles de neurotoxicité des BPC non-coplanaires impliquerait l'altération de la voie de signalisation calcique au niveau des cellules musculaires et neuronales chez les mammifères (Kodavanti et al., 1996; Llansola et al., 2010; Pessah et al., 2006). La forte affinité des BPC non-coplanaires avec les récepteurs de la ryanodine qui régulent les échanges et le relargage du calcium expliquerait cette altération de la voie calcique (Kodavanti et al., 1995; Wong et al., 1997). Les récepteurs de la ryanodine régulent de nombreux processus physiologiques et pathophysiologiques au niveau du système nerveux central et périphérique, ainsi que plusieurs mécanismes biologiques fondamentaux tels que la contraction musculaire et la croissance neuronale (Pessah et al., 2006, 2010). Initialement observée chez les mammifères, l'altération de l'activité des récepteurs de la ryanodine a récemment été démontrée in vitro chez les cellules musculaires de truite arc-en-ciel (Fritsch et Pessah, 2013).

En dépit des preuves d'effets neurotoxiques précédemment cités de ces composés chez les mammifères, très peu d'études ont été publiées chez les poissons, tout particulièrement concernant leurs effets neurotoxiques et embryotoxiques chez les jeunes stades de vie à des doses réalistes d'un point de vue environnemental. À notre connaissance, il existe une seule étude de ce type : suite à l'exposition de poissons zèbre adultes à de la nourriture contaminée avec un mélange réaliste (concentrations comparables à celles retrouvées dans la Seine ou

la Loire, France) de quatre BPC non-coplanaires, Péan et al. (2013) ont mis en évidence des perturbations comportementales (comportement nocturne modifié, hyperactivité dans une situation de stress, modification du positionnement vertical dans la colonne d'eau) aussi bien chez ces individus adultes que chez leur juvéniles.

Les mélanges techniques de BPC

Les biphényles polychlorés ont été majoritairement produits, commercialisés et utilisés par l'industrie sous forme de mélanges techniques de plusieurs congénères. Le plus connu d'entre eux est probablement l'Aroclor, produit par Monsanto jusqu'à la fin des années 1970 (Frame, 1999). Le tableau 2 dresse une liste des études récentes (de 2003 à aujourd'hui) en rapport avec les effets embryotoxiques et neurotoxiques de quelques mélanges techniques de BPC chez les jeunes stades de vie (embryons et larves) des poissons. Ces études sont à la fois peu nombreuses, et pour la plupart (4 études sur 6) centrées sur des effets embryotoxiques sévères telles que la mortalité, des malformations ou une réduction du succès d'éclosion. De plus, là aussi la balnéation des embryons dans une solution contenant le mélange technique est le plus souvent utilisée : puisque tous les congénères de BPC n'ont pas le même coefficient de partage octanol/eau (Han et al., 2006), la pénétration de chacun d'entre eux à travers le chorion diffère et l'embryon n'est pas exposé aux différents congénères de BPC dans les mêmes proportions que celle du mélange technique. Puisque le plus souvent le mélange technique n'est pas caractérisé chimiquement et que les concentrations en BPC ne sont pas non plus mesurées dans les tissus des larves ou juvéniles à la fin de l'exposition, il est difficile de savoir quels congénères (coplanaires ou non) sont responsables des effets embryotoxiques et/ou neurotoxiques observés.

La composition d'un mélange technique de BPC peut varier sensiblement d'un lot de production à l'autre, et pour cette raison la caractérisation chimique d'un mélange technique devrait toujours être réalisée lorsque ce dernier est utilisé au cours d'expériences de toxicité (Burgin et al., 2001). L'exemple le plus documenté à ce sujet est probablement celui de l'Aro-

Tableau 2: Études récentes (de 2003 à 2014) en rapport avec les effets embryotoxiques et neurotoxiques de quelques mélanges techniques de BPC chez les jeunes stades de vie (embryons et larves) des poissons.

Mélange	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Caractérisation chimique du mélange	Référence
Aroclor 1254	↘Croissance, Altération réponse de fuite	*Micropogonias undulatus*	Diète (adultes)	0.4 mg kg^{-1} de poisson par jour	0.66 µg œuf^{-1}	Non	McCarthy et al. (2003)
Kanechlor-400	↘Succès d'éclosion	Medaka (*Oryzias latipes*)	Diète (adultes)	0.75 µg g^{-1} de poisson par jour	22 ng g^{-1}	Non	Nakayama et al. (2005)
Aroclor 1254	↘Croissance neuronale, ↘Concentration sérotonine, ↘Expression gènes liés aux neurones	Poisson zèbre (*Danio rerio*)	Balnéation (acétone)	100 ppm	n.d.[a]	Non	Kreiling et al. (2007)
Aroclor 1254	Mortalité, Syndrome MSB[b], Courbatures dorsales	Poisson zèbre (*Danio rerio*)	Balnéation (méthanol)	0.125-1.0 mg L^{-1}	n.d.	Non	Ju et al. (2012)

Tableau 2 (suite)

Mélange	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Caractérisation chimique du mélange	Référence
Aroclor 1254	Malformations (non décrites)	Carpe de Prusse (*Carassius gibelio*) et commune (*Cyprinus carpio*)	Balnéation	1-100 ng mL^{-1}	n.d.	Non	Socha et al. (2012)
Aroclor 1254	Mortalité, ↘Succès d'éclosion, Syndrome MSB[b]	*Paralichthys olivaceus*	Balnéation	1-40 µg L^{-1}	n.d.	Non	Min et Kang (2013)

[a] Non déterminé par le ou les auteur(s).

[b] Syndrome de la maladie du sac bleu. Voir texte pour détails.

Le signe ↗ est utilisé pour signifier une augmentation

Le signe ↘ est utilisé pour signifier une diminution

clor 1254. En raison d'un changement dans son mode de production de la part de Monsanto, deux types d'Aroclor 1254 ont été historiquement disponibles sur le marché (Frame, 1999; Johnson et al., 2008). Le second type, appelé production tardive d'Aroclor 1254, a été produit dès 1971 à partir d'un résidu de production de l'Aroclor 1016 nommé Montar. Il contenait beaucoup plus de BPC coplanaires, de traces de PCDD et de PCDF que l'Aroclor 1254 typique, ou de production initiale, produit de 1929 à 1970 (Frame, 1999; Johnson et al., 2008). Cet Aroclor 1254 typique a compté pour plus de 99% de la production totale d'Aroclor 1254 par Monsanto et a donc été vraisemblablement le type le plus introduit dans l'environnement. Pourtant, étant donné sa disponibilité au cours des dernières années de production, au moment où la communauté scientifique a commencé à s'intéresser aux effets toxiques des BPC, c'est le type issu de la production tardive d'Aroclor 1254, plus toxique (Burgin et al., 2001), qui a été majoritairement étudié, conduisant à une surévaluation possible des risques environnementaux associés aux mélanges techniques de BPC (Frame, 1999).

Les pesticides organochlorés

Les pesticides organochlorés regroupent de nombreux composés d'origine anthropique introduits volontairement et massivement dans l'environnement pour éliminer les parasites des cultures, contrôler certaines pandémies véhiculées par des insectes (malaria, typhus, etc.) ou encore pour certains usages pharmaceutiques. On peut citer par exemple le DDT (dichlorodiphényltrichloroéthane), le lindane (aussi connu sous le nom d'hexachlorocyclohexane, ou HCH), le chlordane, la dieldrine ou le mirex. Leurs effets neurotoxiques, possiblement responsables du déclin de certaines espèces d'oiseaux, sont bien documentés chez ces derniers (Walker, 2003). Ces effets incluent tremblements, convulsion, réduction de la concentration de certains neurotransmetteurs (sérotonine, dopamine, noradrénaline, etc.), ainsi qu'une variété d'altérations comportementales pouvant mener directement ou indirectement à des effets néfastes à l'échelle de la population, en influençant négativement la capacité de prédation, la capacité d'échapper aux prédateurs, ou encore la capacité de reproduction des individus

(Walker, 2003).

La littérature concernant leurs effets embryotoxiques et neurotoxiques chez les poissons, particulièrement les jeunes stades de vie, est en revanche plus limitée. Quelques études *in vivo* ou *in vitro* ont démontré que certains pesticides organochlorés historiques tels que le DDT, le lindane ou le chlordane pouvaient causer des effets neurotoxiques aussi chez des poissons adultes (Anderson et Peterson, 1969; Anderson et Prins, 1970; Bhattacharjee et Das, 2013; Gooch et al., 1990). Concernant les jeunes stades de vie, des embryons de dorade royale (*Sparus aurata*) exposés à du lindane par balnéation (0.1-10 mg^{-1}) ont montré une mortalité importante dès 1 mg L^{-1}, ainsi que des altérations comportementales telles qu'une diminution de la vitesse de nage, une incapacité à répondre à un stimulus externe, des mouvements non coordonnés et des tremblements (Oliva et al., 2008). Des embryons de poisson zèbre exposés à des doses élevées, mais réalistes, de DDT (entre 18.000 et 62.000 ng g^{-1} de poids humide de tissu, des concentrations comparables à celles mesurées chez certains mammifères marins) ont montré des altérations comportementales telles que de l'hyperactivité, des convulsions, des tremblements ou encore une paralysie ou une absence de réponse aux stimuli (Tiedeken et Ramsdell, 2009).

Les diphényléthers polybromés

Les PBDE sont des composés d'origine anthropique principalement utilisés comme retardateurs de flamme dans un grand nombre de matériaux plastiques et textiles. Leur structure chimique (figure 7) comprend deux cycles benzènes reliés par un atome d'oxygène (O). Structurellement proches des BPC, 209 congénères sont théoriquement possibles, mais seulement une quarantaine sont retrouvés de routine dans l'environnement (Ross et al., 2009). Tout comme les BPC, ces composés sont persistants dans l'environnement et bioaccumulés par les organismes marins. Les concentrations de ces composés dans le *biota* semblent augmenter depuis plusieurs décennies, y compris dans les Grands Lacs (Batterman et al., 2007), leur attribuant le statut de contaminants émergents, par opposition aux contaminants histo-

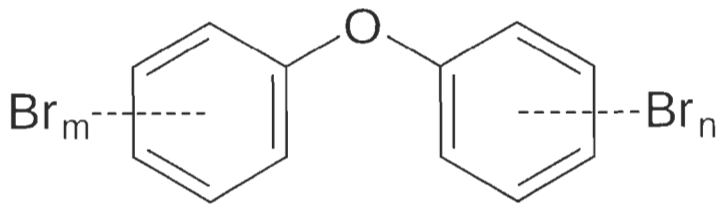

Figure 7: Structure générale des PBDE.

riques que sont les dioxines et composés apparentés, par exemple. En dépit de leur analogie structurelle avec les BPC, ces composés n'ont qu'une faible affinité avec le récepteur Ah et il est admis qu'ils n'induisent pas de réponses toxiques du type de celles des dioxines (Chen et Bunce, 2003). Les PBDE peuvent causer divers effets toxiques en perturbant entre autres les niveaux d'hormones thyroïdiennes, notamment T4, chez les mammifères et les oiseaux au cours de leur développement ou au stade adulte (Fernie et al., 2005; Hallgren et al., 2001; Zhou et al., 2002). Ces perturbations peuvent être critiques lors du développement embryonnaire des mammifères et des oiseaux, puisque les hormones thyroïdiennes jouent un rôle essentiel dans le développement cérébral et la mise en place du système nerveux central (Koibuchi et Chin, 2000). Les hormones thyroïdiennes jouent aussi un rôle prépondérant dans le développement embryonnaire des poissons (Power et al., 2001). Chez les mammifères, les PBDE sont également susceptibles d'induire des effets neurotoxiques en provoquant du stress oxydant, de l'apoptose et des dommages à l'ADN (He et al., 2008).

Le tableau 3 dresse une liste des études récentes (de 2006 à aujourd'hui) en rapport avec les effets embryotoxiques et neurotoxiques des congénères individuels de PBDE ou de mélanges techniques commerciaux de PBDE chez les jeunes stades de vie (embryons et larves) des poissons. Certaines de ces études ont mis en évidence des effets neurotoxiques à des doses non nécessairement associées à de la mortalité ou des à malformations sévères. Comme dans le cas des études répertoriées pour les dioxines et composés apparentés (tableau 1), la majorité des études listées dans le tableau 3 (10 études sur 11) emploient la

balnéation comme mode d'exposition. Dans les quelques cas (6 études sur 11) où les auteurs fournissent les concentrations internes en PBDE mesurées dans les tissus des embryons ou larves de poissons exposés, ces dernières atteignent régulièrement quelques milliers voir même une dizaine de milliers de ng g^{-1} de poids humide (p.h.) de tissu. Ces concentrations sont nettement plus élevées que les concentrations mesurées dans les tissus de touladi ou d'anguille d'Amérique du lac Ontario dans les années 2000, qui se situaient en dessous de 100 ng g^{-1} p.h. (Batterman et al., 2007; Byer et al., 2013b; Carlson et al., 2010). La littérature concernant l'effet des PBDE sur le développement embryonnaire des poissons à des concentrations et des modes d'exposition réalistes d'un point de vue environnemental semble faire défaut.

Les mélanges organiques complexes : approches possibles

Comme énoncé précédemment, les anguilles du lac Ontario accumulent différents POP (PCDD, PCDF, BPC, PBDE, etc.) et leurs embryons sont donc exposés à un mélange complexe de contaminants. Dans la littérature, il existe plusieurs exemples d'études concernant les effets embryotoxiques de mélanges organiques complexes extraits de tissus de poissons, tout particulièrement dans le cas des salmonidés du lac Ontario (Harris et al., 1994; Walker et al., 1996a; Wilson et Tillitt, 1996; Wright et Tillitt, 1999). Certaines d'entre elles ont démontré que le concept d'EQT-TCDD prédisait correctement l'embryotoxicité observée (Walker et al., 1996a; Wright et Tillitt, 1999). Ceci suggère qu'une caractérisation chimique de ces mélanges, sans exposition d'embryons, aurait théoriquement suffit à prédire l'embryotoxicité observée. Néanmoins, cela ne semble pas toujours être le cas : par exemple, Harris et al. (1994) ont démontré que les composés moins toxiques présent dans un mélange organique complexe, en l'occurrence les BPC non-coplanaires, pouvaient atténuer la toxicité des composés plus nocifs tels que les PCDD et les PCDF. Le concept d'EQT-TCDD, qui chez les poissons est largement basé sur des études d'embryo-mortalité chez les salmonidés, ne tient pas compte de la toxicité des autres POP cités précédemment (BPC non-coplanaires, PBDE,

Tableau 3: Études récentes (de 2006 à 2014) en rapport avec les effets embryotoxiques et neurotoxiques des PBDE (congénères individuels ou mélanges techniques commerciaux) chez les jeunes stades de vie (embryons et larves) des poissons.

Composé	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Référence
DE-71*	↘Activité locomotrice, ↘Capacité prédation, Altération réponse de fuite	Choquemort (*Fundulus heteroclitus*)	Balnéation (DMSO[a])	0.001-100 µg L^{-1}	n.d.[b]	Timme-Laragy et al. (2006)
PBDE 47	↘Succès d'éclosion, ↘Croissance, Courbatures dorsales, Problèmes cardiaques	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	100-5000 µg L^{-1}	n.d.	Lema et al. (2007)
PBDE 47	↘Activité locomotrice	Poisson zèbre (*Danio rerio*)	Diète (adultes)	10-1000 ng g^{-1} p.h.[c] de nourriture	2.7-410 ng g^{-1} p.h.	Chou et al. (2010)
PBDE 209	↘Croissance, ↘Activité locomotrice, Hyperactivité	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	0.001-1.0 µM	200-4000 ng g^{-1} p.h.	He et al. (2011)

Tableau 3 (suite)

Composé	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Référence
PBDE 28, 47, 99, 100, 153 et 183	Mortalité, Courbatures dorsales, Hyperactivité	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	0.635-20 mg L^{-1}	n.d.	Usenko et al. (2011)
PBDE 49	Mortalité, Courbatures dorsales, Altération réponse de fuite	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	4-32 µM	n.d.	McClain et al (2012)
DE-71*	↘Activité locomotrice, ↘Activité acétylcholinestérase, ↘Expression gènes impliqués dans développement du SNC	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	100-1000 µg L^{-1}	6100-17500 ng g^{-1} p.h.	Chen et al. (2012a)

Tableau 3 (suite)

Composé	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Référence
DE-71*	↘Activité locomotrice, ↘Activité acétylcholinestérase, ↘Expression gènes impliqués dans développement du SNC[d]	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	0.16-4.0 µg L^{-1}	100-6000 ng g^{-1} p.h.	Chen et al. (2012b)
PBDE 47	↘Activité locomotrice, Altération réponse de fuite, ↘Croissance neuronale	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	1.25-20 µM	40-240 µg g^{-1} p.h.	Chen et al. (2012c)
DE-71*	↘Nombre celllules ganglionnaires rétine, ↘Vision	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	1-100 µg L^{-1}	1-100 µg g^{-1} p.h.	Chen et al. (2013)

Tableau 3 (suite)

Composé	Effets	Espèce	Exposition (solvant)	Doses nominales	Doses internes	Référence
PBDE 47	↘Activité locomotrice	Poisson zèbre (*Danio rerio*)	Balnéation (DMSO)	5-500 µg L^{-1}	n.d.	Zhao et al. (2014)

[*] Mélange technique.

[a] Diméthylsulfoxyde.

[b] Non déterminé par le ou les auteur(s).

[c] Poids humide

[d] Système nerveux central

Le signe ↗ est utilisé pour signifier une augmentation

Le signe ↘ est utilisé pour signifier une diminution

etc.), des POP émergents mesurés dans les tissus de touladis du lac Ontario (Clement et al., 2012) dont la présente thèse ne traite pas, ainsi que de leurs interactions possibles avec les composés apparentés aux dioxines. Ainsi, au cours de la présente thèse, afin d'évaluer le potentiel embryotoxique de mélanges complexes de POP accumulés par l'anguille d'Amérique du lac Ontario entre 1988 et 2008, le choix a été fait de coupler analyses chimiques (Byer, 2013) et exposition *in vivo* d'embryons.

Hypothèses de recherche, démarche expérimentale générale et objectifs

Hypothèses de recherche

La présente thèse a fait partie intégrante d'un vaste projet financé par le CRSNG (Conseil de Recherches en Sciences Naturelles et en Génie du Canada) et coordonné par le Dr. Peter Hodson (Queen's University, département de biologie). Ce projet a regroupé plusieurs études *in vitro* (sur des cellules d'anguilles) et *in vivo* (sur des poissons modèles tels que le médaka japonais et le choquemort, mais également sur des anguilles jaunes) dont le but commun était d'améliorer la compréhension du rôle possible des polluants organiques persistants dans le déclin de l'anguille d'Amérique (Byer, 2013; Byer et al., 2013a,b, in press; Kennedy, 2010). Ainsi, la présente thèse a eu pour objectif de répondre aux trois hypothèses suivantes :

H1 – Les POP individuels retrouvés dans les tissus des anguilles ainsi qu'un mélange technique de BPC, l'Aroclor 1254,

— n'ont pas la même toxicité chez les embryons d'un poisson modèle, *Fundulus heteroclitus*, à des concentrations réalistes d'un point de vue environnemental.

— ne produisent pas le même patron de réponses de biomarqueurs chez ce poisson modèle.

H2 – Des extraits organiques tissulaires préparés à partir d'anguilles capturées au sein du lac Ontario entre 1988 et 2008,

— n'ont pas la même toxicité chez les embryons d'un poisson modèle, *F. heteroclitus*.
— ne produisent pas le même patron de réponses de biomarqueurs chez ce poisson modèle.

H3 – L'embryotoxicité chez *F. heteroclitus* des extraits d'anguille ne peut pas être prédite par leur concentration en EQT-TCDD ; le patron de réponses de biomarqueurs diffère de celui observé avec les composés apparentés aux dioxines, à une concentration en EQT-TCDD équivalente.

Choix de l'espèce modèle *Fundulus heteroclitus*

Puisqu'il est techniquement difficile de travailler directement sur des embryons d'anguilles (mortalité élevée des embryons en captivité, reproduction induite de manière artificielle, etc.), les embryons d'un poisson euryhalin modèle présent dans le golfe du Saint-Laurent, le choquemort (*Fundulus heteroclitus*), ont été utilisés au cours des tests d'embryotoxicité. Le choquemort est l'un des poissons le plus représenté au sein des marais intertidaux situé le long de la côte nord-américaine, où il joue un rôle clé en tant que prédateur piscivore mais également comme proie de nombreuses autres espèces (Able et al., 2006). La bonne connaissance de son développement embryonnaire (Armstrong et Child, 1965), la relative facilité de la maintenir et de la faire se reproduire en captivité, ainsi que les avancées récentes concernant le séquençage de son génome en font une espèce de choix pour les études en laboratoire en général et les expériences d'embryotoxicité en particulier (Burnett et al., 2007) (tableau 1). Il a été utilisé avec succès dans le cadre d'expériences d'embryotoxicité au sein des installations de l'Institut Maurice-Lamontagne pendant plusieurs années (Couillard, 2002; Couillard et al., 2008, 2011; Fortin et al., 2008).

Choix de la méthode d'exposition des embryons

Quel que soit le contaminant ou mélange de contaminants employé au cours de la présente thèse, les embryons de *F. heteroclitus* ont été exposés par injection intravitelline. Cette méthode a été choisie plutôt que la balnéation, par exemple, afin de simuler au mieux le transfert maternel des contaminants, de s'affranchir des problèmes de pénétration des contaminants à travers le chorion et ainsi d'exposer les embryons aux contaminants dans des proportions les plus fidèles possible à celles du mélange initial, dans le cas d'un mélange technique ou d'extraits organiques tissulaires. La contamination des embryons est réalisée à l'aide d'un Eppendorf FemtoJet® express microinjector couplé à un stéréomicroscope (figure 8). Le volume injecté par embryon est compris entre 2 et 10 nL : ce volume est estimé en mesurant le diamètre de la goutte injectée à l'aide d'un objectif gradué. Les contaminants ou mélanges de contaminants auront préalablement été mis en solution dans de la trioléine. La trioléine est un triglycéride naturel, préalablement utilisé avec succès comme solvant au cours d'expérience d'embryotoxicité chez les poissons (Carvalho et Tillitt, 2004; Wilson et Tillitt, 1996).

Objectifs

La présente thèse est séparée en trois chapitres (ou articles) distincts qui répondent à trois objectifs ou groupes d'objectifs précis. Le **premier objectif** (article 1, publié dans *Aquatic Toxicology* en 2013) se concentre sur les effets embryotoxiques et neurotoxiques sous-létaux chez *F. heteroclitus* de deux composés apparentés aux dioxines, la 2,3,7,8-tétrachlorodibenzo-*p*-dioxine (TCDD) et le 3,3',4,4',5-pentachlorobiphényle (BPC126). Ces deux composés sont parmi les composés apparentés aux dioxines les plus toxiques chez les poissons (Van den Berg et al., 1998) et sont les deux composés principaux suspectés d'avoir contribué au déclin du touladi dans le lac Ontario au cours des années 1960 et 1970 (Cook et al., 2003). On s'attendait donc à les retrouver dans les extraits tissulaires des anguilles du lac Ontario et à ce qu'ils contribuent à l'embryotoxicité de ces derniers observée chez *F.*

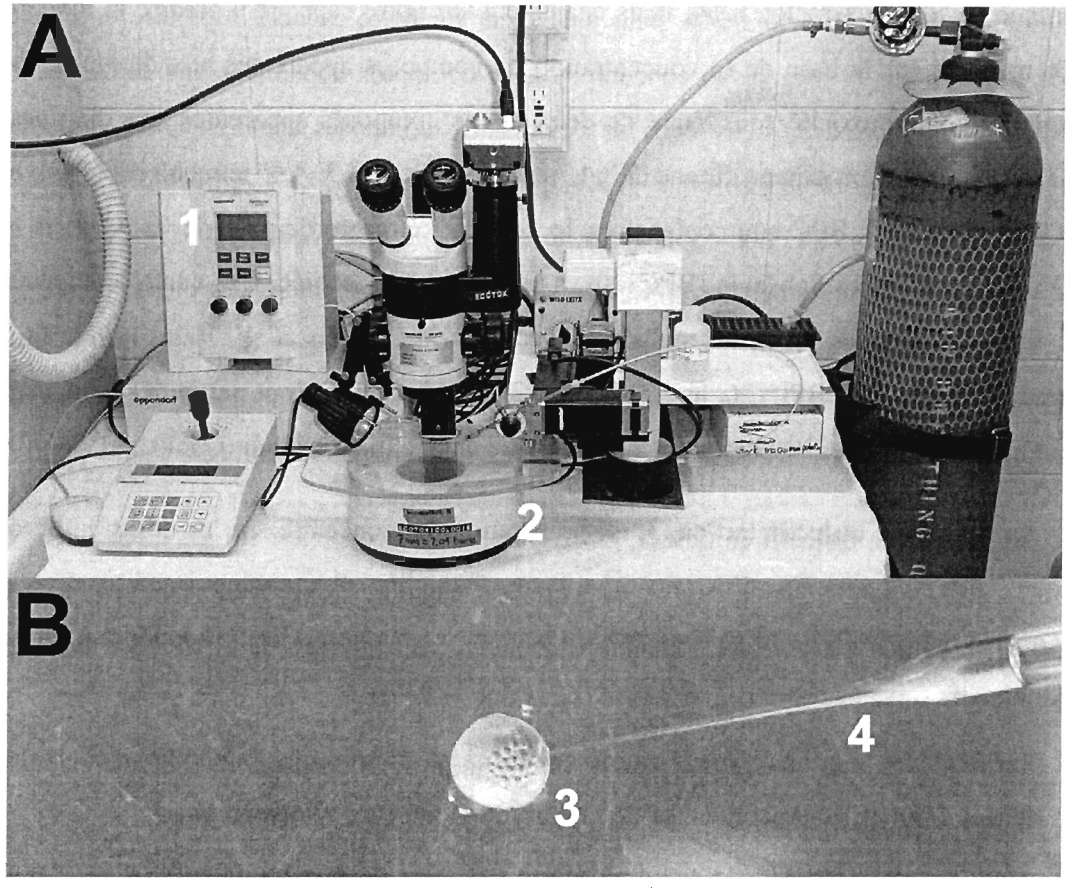

Figure 8: Système d'injection utilisé : (A) Eppendorf FemtoJet® express microinjector (1) couplé à un stéréomicroscope (loupe binoculaire) (2). (B) Plan rapproché d'un embryon de *F. heteroclitus* (3) pendant l'injection avec une aiguille de quartz remplie d'une solution de trioléine (4).

heteroclitus.

Le **second objectif** (article 2, publié dans *Aquatic Toxicology* en 2014) se concentre sur les effets embryotoxiques et neurotoxiques sous-létaux chez *F. heteroclitus* d'un mélange technique de BPC, l'Aroclor 1254, mais également sur notre capacité à prédire la toxicité de ce mélange sur la base de sa concentration en composés apparentés aux dioxines. En parallèle, l'embryotoxicité sous-létale de deux autres composés apparentés aux dioxines, le 2,3,4,7,8-pentachlorodibenzofurane (2,3,4,7,8-PnCDF) et le 3,3',4,4'-tetrachlorobiphényle (BPC77), et de deux BPC non coplanaires, le 2,2',5,5'-tetrachlorobiphényle (BPC52) et le 2,3,3',4',6-pentachlorobiphényle (BPC110), a également été étudiée. Ces quatre composés sont retrouvés dans les tissus des touladis du lac Ontario (Huestis et al., 1996, 1997). Tout comme la TCDD et le BPC126, le 2,3,4,7,8-PnCDF et le BPC77 font partie des composés apparentés aux dioxines suspectés d'avoir contribué au déclin du touladi (Cook et al., 2003).

Le **troisième objectif** (article 3, en préparation) se concentre sur les effets embryotoxiques chez *F. heteroclitus* d'extraits organiques d'anguilles d'Amérique capturées dans le lac Ontario entre 1988 et 2008. Plus spécifiquement, ce chapitre vise à répondre aux trois sous-objectifs suivants :

1. Vérifier si les anguilles du lac Ontario ont accumulé suffisamment de POP au cours des années 1980 et 1990 pour causer de l'embryotoxicité chez *F. heteroclitus*.
2. Vérifier si l'embryotoxicité de ces extraits varie entre 1988 et 2008.
3. Tester notre capacité à prédire l'embryotoxicité de ces extraits à partir de leur caractérisation chimique, en utilisant les connaissances acquises au cours des chapitres 1 et 2.

ARTICLE I

RELATIVE POTENCY OF PCB126 TO TCDD FOR SUBLETHAL EMBRYOTOXICITY IN THE MUMMICHOG (*FUNDULUS HETEROCLITUS*)

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Contribution de chaque auteur - **C. Rigaud** : conception du projet, expérimentation et analyse des données, rédaction de l'article et conception des figures ; **C. M. Couillard** : conception du projet, supervision, aide à l'expérimentation et à la rédaction ; **J. Pellerin** : supervision et aide à la rédaction ; **B. Légaré** : aide à l'expérimentation ; **P. Gonzalez** : supervision et aide à l'expérimentation de la partie biologie moléculaire ; **P. V. Hodson** : direction et coordination du projet CRSNG, supervision et aide à la rédaction.

1.1 Résumé

Le potentiel toxique relatif (PTR) du 3,3',4,4',5-pentachlorobiphényle (BPC126) par rapport à la 2,3,7,8-tetrachlorodibenzo-*p*-dioxine (TCDD) a été évalué pour des réponses sous-létales chez les embryons de *Fundulus heteroclitus*. Les œufs ont subi des injections intravitellines de doses sous-létales de BPC126 (312-5000 pg g^{-1} de poids humide, p.h.) ou de TCDD (5-1280 pg g^{-1} p.h.). À 16 jours post-fécondation (JPF), des malformations crânio-faciales ont été observées chez les larves issues des œufs traités avec les deux plus hautes doses de BPC126 (2500-5000 pg g^{-1} p.h.). Les deux composés ont causé une réduction dose-dépendante de la croissance larvaire et de la capacité de prédation (dès 1250 pg g^{-1} p.h.), ainsi qu'une induction de l'activité de l'ethoxyrésorufine-*O*-dééthylase (EROD) (dès 80 pg g^{-1} p.h.). Les relations dose-réponse pour l'activité EROD pour la TCDD et le BPC126 présentant des pentes similaires, le PTR du BPC126 par rapport à la TCDD a pu être estimé à 0.71. C'est 140 fois supérieur au facteur d'équivalence toxique (FET) de référence de l'Organisation Mondiale de la Santé (OMS) pour le BPC126 chez les poissons (0.005), qui est basé sur des données d'embryo-mortalité obtenues chez la truite arc-en-ciel (*Oncorhynchus mykiss*). La pente de la relation dose-réponse pour la capacité de prédation était plus prononcée pour le BPC126 par rapport à la TCDD, suggérant des mécanismes d'action différents. Le niveau d'expression de plusieurs gènes a également été mesuré par réaction en chaîne par polymérase quantitative (qPCR) à la suite d'expositions à des doses uniques de TCDD ou de BPC126 (1280 et 1250 pg g^{-1} p.h., respectivement) causant un même niveau d'induction EROD. Un patron de réponses différent a été observé entre la TCDD et le BPC126 : ce dernier a semblé induire des réponses anti-oxidantes par l'intermédiaire du gène *sod2*, mais pas la TCDD. Ces résultats suggèrent que les PTR sont spécifiques à chaque espèce et que le FET actuel du BPC126 pourrait sous-estimer son potentiel toxique chez certaines espèces de poissons. Il est recommandé de développer des PTR spécifiques à chaque espèce pour une variété de réponses sous-létales à des doses réalistes d'un point de vue environnemental.

1.2 Abstract

The relative potency (ReP) of 3,3',4,4',5-pentachlorobiphenyl (PCB126) to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for sublethal responses was assessed in *Fundulus heteroclitus* embryos. Eggs were treated with intravitelline injections of graded sublethal doses of PCB126 (312-5000 pg g^{-1} wet weight, ww) or TCDD (5-1280 pg g^{-1} ww). At 16 days post-fertilization (DPF), craniofacial deformities were observed in larvae hatched from eggs treated with the two highest doses of PCB126 (2500-5000 pg g^{-1} ww). Both compounds caused a dose-responsive reduction of larval growth and prey capture ability (at ≥1250 pg g^{-1} ww), and induction of ethoxyresorufin-*O*-deethylase (EROD) activity (at ≥80 pg g^{-1} ww). The dose-response relationships for EROD activity for PCB126 and TCDD had similar slopes and the ReP of PCB126 to TCDD for EROD activity was estimated at 0.71. This is 140-fold higher than the World Health Organization (WHO) TCDD equivalency factor (TEF) of PCB126 for fish (0.005), which is based on rainbow trout (*Oncorhynchus mykiss*) embryolethality data. The slope of the dose-response relationship for prey capture ability for PCB126 was steeper than for TCDD, suggesting different mechanisms of action. Expression levels of several genes were also studied by quantitative real-time polymerase chain reaction (qPCR) following exposure to single doses of TCDD or PCB126 (1280 and 1250 pg g^{-1} ww, respectively) causing similar EROD induction. A different pattern of responses was observed between PCB126 and TCDD: PCB126 appeared to induce antioxidant responses by inducing *sod2* expression, while TCDD did not. These results suggest that relative potencies are species-specific and that the current ReP for PCB126 underestimates its toxicity for some fish species. It is recommended to develop species-specific RePs for a variety of sublethal endpoints and at environmentally relevant doses.

1.3 Introduction

Planar halogenated hydrocarbons (PHHs) include polychlorinated dibenzo-*p*-dioxins (PCDDs), -furans (PCDFs) and coplanar polychlorinated biphenyls (PCBs). These dioxin-like compounds (DLCs) are persistent and widespread pollutants that accumulate in fish tissues due to their lipophilic properties. Responses of early life stages of fish to PHHs include induction of cytochrome P4501A (CYP1A) detoxification enzymes, developmental arrest, craniofacial deformities, pericardial and yolk sac edemas, hemorrhages and mortality (Belair et al., 2001; Henry et al., 1997; Hill et al., 2004a,b; Teraoka et al., 2002). Lethal embryotoxicity of PHHs may have contributed to lake trout (*Salvelinus namaycush*) decline in Lake Ontario, Canada, between 1940 and 1980 (Cook et al., 2003). Following cessation of mass production in the 1970s (Ross, 2004), concentrations of PHHs declined in Lake Ontario (Gewurtz et al., 2009) and survival of lake trout embryos improved (Cook et al., 2003). However, sublethal effects of PHHs on early life stages such as growth retardation (Carvalho et al., 2004) and impaired prey capture ability (Carvalho and Tillitt, 2004) may still impact lake trout population today.

Biological responses to PHHs are modulated by the aryl hydrocarbon receptor (AHR) (Poland and Glover, 1976), a highly conserved cytosolic receptor among vertebrates (Hahn and Karchner, 1995). Teleosts possess at least two isoforms, AHR1 and AHR2 (Hahn et al., 2006). Affinity of PHHs for AHR is greater when their chlorines are located on lateral position, i.e. when their structure is similar to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Safe, 1990), considered to be the most toxic PHH. The toxic potency of complex mixtures of PHHs can be predicted from their TCDD toxic equivalents concentration (TCDD-TEQs), i.e. the sum of each PHH concentration times it toxic equivalent factor (TEF; relative toxicity of each PHH versus TCDD) (van Zorge et al., 1989). TEFs are global values decided by consensus using data from multiple experiments and endpoints, which generate more specific values called relative toxic potencies, or RePs (Jensen et al., 2010).

TEFs for fish have not been updated since 1998 and are mainly based on embryolethality studies of salmonids exposed by nano-injection (Van den Berg et al., 1998; Walker and Peterson, 1991; Zabel et al., 1995b). For example, the World Health Organization (WHO) reference TEF in fish for the most toxic coplanar PCB, 3,3',4,4',5-pentachlorobiphenyl (PCB126), is 0.005 (Van den Berg et al., 1998). However, some studies suggest a higher toxic potency for PCB126 in non-salmonid species. For instance, the lethal embryotoxicity of waterborne PCB126 was said to be 0.032 times that of TCDD in Japanese medaka (*Oryzias latipes*) (Kim and Cooper, 1999). To our knowledge, relative toxic potencies of PHHs were never assessed in marine fish species. Moreover, toxic potencies of PHHs may vary according to the dose: non-parallel dose-response curves among various PCDDs and PCDFs versus TCDD lead to higher toxic potencies for CYP1A induction at low doses compared to high doses (Parrott, 1997) in rainbow trout (*Oncorhynchus mykiss*). Thus, toxicity data from more fish species and endpoints, and a wider dose range are required to perform relevant risk assessments for PHHs.

In this study, the relative potency of PCB126 and TCDD for sublethal responses was assessed in a marine model fish, the mummichog (*Fundulus heteroclitus*) (Burnett et al., 2007). Exposure of early life stages of mummichog to lethal aqueous concentrations (≥2100 ng L^{-1}, leading to an internal dose of 745 ng g^{-1} dry weight) of PCB126 induces developmental abnormalities including pericardial edema and tail hemorrhages characteristic of the blue sac disease (BSD) syndrome observed in *F. heteroclitus* and other fish species exposed to TCDD (Nacci et al., 1998, 2002; Prince and Cooper, 1995; Walker et al., 1991). Exposure of mummichog embryos to sublethal doses (up to 50 pg egg^{-1}) of PCB126 applied on the surface of the eggshell reduced body length and prey capture ability and caused induction of ethoxyresorufin-*O*-deethylase (EROD) activity at doses causing no morphological abnormalities (Couillard et al., 2011). Intravitelline injection is the preferred route of exposure to estimate relative potencies because the proportion of chemical reaching embryo tissues after topical treatment or aqueous exposure can differ among PHHs. In the present study, basal locomotor activity and prey capture ability were measured in parallel to more classi-

cal AHR-related endpoints (EROD induction, malformations and developmental retardation) in mummichog embryos injected with TCDD and PCB126. Relative quantitative polymerase chain reaction (qPCR) was used to quantify the expression of specific target genes to compare mechanistic pathways between PCB126 and TCDD.

1.4 Materials and methods

1.4.1 Chemicals preparation and analyses

TCDD (in acetone, >98% purity) and PCB126 (solid form, 99% purity) were purchased from Wellington Laboratories (Guelph, ON, Canada) and Ultra Scientific (North Kingstown, RI, USA), respectively. Sigma-Aldrich (St-Louis, MO, USA) supplied the triolein. Triolein is an unsaturated triglyceride and was previously and successfully used as a vehicle solvent for PHHs in fish embryotoxicity studies (Carvalho and Tillitt, 2004; Wilson and Tillitt, 1996). The LD_{50} (median lethal dose) of TCDD for nano-injected lake trout were 81 (76-101) pg g^{-1} (Wright, 2006) and 80 (68-91) pg g^{-1} (Walker et al., 1994) using triolein or liposomes as carriers, respectively (mean and 95% confidence limits).

Stock solutions of TCDD, previously separated from acetone by evaporation, and PCB126 were prepared by dissolving a known mass of each compound in triolein to reach nominal concentrations of 15 ng μL^{-1} and 1 μg μL^{-1}, respectively. Concentrations were validated with low (PCB126) or high (TCDD) resolution gas chromatography-mass spectrometry (GC-MS) in Laboratories of Expertise in Aquatic Chemical Analyses (LEACA, Fisheries and Oceans Canada, Maurice-Lamontagne Institute, QC, and Institute of Ocean Sciences, BC, Canada). The concentration of PCB126 in a working solution of 12.5 ng μL^{-1} was validated by low resolution GC-MS. The purity of the triolein control was assessed by high resolution GC-MS for PCDDs, PCDFs and both coplanar and mono-ortho-substituted PCBs.

1.4.2 Spawners and eggs handling

F. heteroclitus adults (200 females and 100 males) were collected in May 2009 in Horton's Creek (Miramichi, NB, Canada). Fish were divided in two equals groups and maintained in two 420 L fiberglass tanks with flowing 25‰ salt water (St. Lawrence Estuary water filtered through silicate sand with a grain of 0.8-1.2 mm), under controlled photoperiod (16 h:8 h). Gonad maturation was stimulated by raising the water temperature progressively from 10°C to 19°C (Boyd and Simmonds, 1974). Spawners were fed with pelleted cichlid food (Nutrafin®, Montréal, QC, Canada) supplemented with dried shrimps and *Tubifex* worms. Eggs were collected on artificial spawning substrates and randomly pooled into Petri dishes. Using a stereo microscope, normal and fertilized eggs between stages 9 and 11 (Armstrong and Child, 1965) were selected for embryotoxicity assays.

1.4.3 Embryos exposure

Serial dilutions of TCDD and PCB126 in triolein were prepared from stock solutions (see section 1.4.1). Normal and fertilized eggs were divided randomly into seven groups of 35 embryos. Embryos were exposed individually by nano-injection of 2 nL of the triolein solutions, directly into the yolk. Thus, each group of embryos was exposed to a known dose of TCDD or PCB126 expressed in pg g^{-1} wet weight (ww, wet embryo mass was approximately 0.005 g). Eggs nested in custom made agarose supports were injected with an Eppendorf FemtoJet® express microinjector (Eppendorf® Canada, Mississauga, ON, Canada) coupled to a stereo microscope (Wilson and Tillitt, 1996). The volume injected was estimated by measuring the diameter of the triolein droplet with an ocular micrometer.

For each compound, the experimental design included two control groups (non-injected and injected with triolein, 0 pg g^{-1} ww) and five groups injected with increasing doses of TCDD (5 to 1280 pg g^{-1} ww) or PCB126 (312 to 5000 pg g^{-1} ww). The doses of TCDD were selected based on results from preliminary studies (unpublished results): *F. heteroclitus*

embryos received intravitelline injections of 25, 250 and 2500 pg g^{-1} ww of TCDD. No mortality was observed at 16 days post-fertilization (DPF) but a significant increase in the prevalence of malformations typical of TCDD toxicity reported in the embryos of various fish species (craniofacial deformities, edemas and hemorrhages). The most severely affected larvae would likely be non viable if observed for a longer period. Based on these preliminary results, we selected a range of doses of TCDD that would be sublethal and would produce effects similar to those observed in embryos exposed to environmentally realistic doses of PCB126.

Following treatment, embryos were distributed randomly into 24-well Costar® polystyrene microplates (Corning Inc. Life Sciences, Lowell, MA, USA) on moist filter paper and incubated for 13 days at 22.5°C under natural photoperiod (14 h:10 h) with daily monitoring for mortality. At 13 DPF, hatching was triggered by adding 25‰ salt water into the wells. After 1 h, unhatched embryos and larvae were transferred individually into 20 mL glass vials containing 15 mL of salt water (25‰) for three more days without feeding and with daily renewal of water (total exposure time = 16 DPF). Mortality and hatching success were monitored daily. Salt water used for embryos and larvae handling was from the same source as described in section 1.4.2 but was additionally UV-sterilized and filtered (0.1 µm pore size). All behavioral assays described further (sections 1.4.4 and 1.4.5) were performed in aerated salt water (25‰), at room temperature.

1.4.4 Locomotor activity

At 16 DPF, 56 larvae (N = 8 per treatment) were distributed randomly in two 48-well Costar® polystyrene microplates (28 larvae per plate, 7 treatments with 4 larvae each). Each plate was placed in succession in a Zebrabox® (ViewPoint Life Sciences Inc., Montréal, QC, Canada) with an automated video-tracking software (Videotrack®, ViewPoint Life Sciences Inc.) to simultaneously record basal locomotor activity of each of the 28 larvae. To detect larval movement, the Videotrack® software compares successive frames in terms of pixels

differences. According to Emran et al. (2008) and after calibration with mummichog larvae, the threshold value for minimum pixel change from frame to frame was set to 4 pixels. After 20 min of acclimation in the dark, basal locomotor activity was recorded during three successive periods of 5 min. The first and third periods were recorded under infrared light. Larvae were exposed to white light during the second 5 min period to monitor their reaction to a visual stimulus (MacPhail et al., 2009). For each larva and each period, raw data provided by the Videotrack® software were expressed as three variables that describe basal locomotor activity: rate of travel (average swimming speed over the total period, mm s^{-1}), active swimming speed (average swimming speed during active time only, mm s^{-1}) and inactivity (% of time the larva spent resting) (Alvarez and Fuiman, 2005).

Mummichog larvae typically stopped moving when light was switched on, and then alternated short movements and resting periods. The number and duration (s) of resting periods (defined as periods of immobility lasting at least 2 s) were counted during the light period. The first resting period, immediately after the light was turned on, was defined as the recovery latency period.

1.4.5 Prey capture ability and efficiency

After the locomotor activity assay, one larva from each of the six treatments (excluding non-injected control group) was randomly distributed in 6 adjacent wells of eight 48-well microplates (N = 8 larvae per treatment). These microplates were prepared in advance by adding 20 *Artemia fransciscana* nauplii in each of the 6 adjacent wells (Couillard et al., 2011; Gahtan et al., 2005). For each larva, predation on nauplii was recorded during 5 min using a digital camera (Canon VIXIA HV30 HD Camcorder®, Canon Canada Inc., ON, Canada) under fluorescent light. Videos were analyzed *a posteriori* to count the number of *Artemia* remaining and the number of feeding strikes each 30 s. The rate of decline of *Artemia* was used as an index of prey capture ability and the number of successful captures per feeding strike was used as an index of prey capture efficiency (Couillard et al., 2011).

1.4.6 Morphometry and biochemistry

After behavioral assays, all larvae were observed under a stereo microscope coupled to an ocular micrometer. Body length was measured and the occurrence of malformations was recorded. The presence or absence of craniofacial deformity was recorded. Craniofacial deformities were characterized by a shortened snout, jaw or forehead (fig. 9). After measurements, 15 unfed larvae per treatment (3 pools of 5 larvae) were frozen in liquid nitrogen and stored at -80°C until EROD activity analyses. EROD activity is a biomarker of CYP1A induction by DLCs (Whyte et al., 2000). EROD activity is estimated by measuring the rate of the CYP1A-mediated deethylation of the substrate 7-ethoxyresorufin (7-ER) to form the product resorufin. EROD activity was measured using an adaptation of a spectrofluorimetric method on microplates from Fragoso et al. (1998), as previously described by Couillard et al. (2008, 2011). Briefly, activity was quantified in the S9 fractions prepared from 3 homogenates of 5 whole mummichog larvae, for each treatment (N = 3). Using a Cytofluor II® plate reader (PerSeptive Biosystems, Framingham, MA, USA), fluorescence of the produced resorufin was measured at 60 s intervals during 13 min and readings were compared to a resorufin standard curve (excitation 530 nm, emission 590 nm). Total protein content of the S9 fraction was also measured with Bradford reagent (BIO-RAD Laboratories, Hercules, CA, USA) and standardized against BSA standards. EROD activity was expressed as pmoles resorufin.min^{-1}.mg^{-1} protein. Measurements were performed in 96-well Costar® polystyrene microplates and a positive control was added in each microplate to assess the quality and repeatability of the measures. Positive controls consisted of S9 fraction of pools of 5 mummichog larvae (14 DPF) exposed during 24 hours to waterborne benzo(a)pyrene (10 $\mu g\ L^{-1}$). EROD activity for 5 positive controls on 5 microplates ranged from 2.41 to 3.28 pmoles resorufin min^{-1} mg^{-1} protein, with a mean value (± standard deviation) equal to 2.89 ± 0.35 pmoles resorufin min^{-1} mg^{-1} protein.

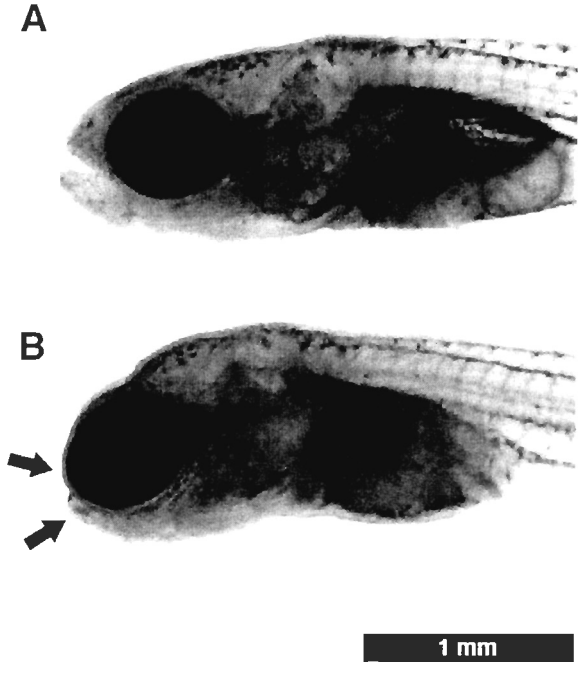

Figure 9: Heads of 16 DPF *F. heteroclitus*: (A) control larva injected with pure triolein with normally developed jaws and (B) larva injected with 5000 pg g^{-1} of PCB126 with atrophy of the upper and lower jaws (arrows).

1.4.7 Relative quantitative polymerase chain reaction (qPCR)

For qPCR analyses, extra samples (N = 30 per treatment) were generated using only one dose selected for each compound: 1280 pg g^{-1} ww and 1250 pg g^{-1} ww for TCDD and PCB126, respectively. These doses were chosen because they caused similar responses in larvae in terms of inducing EROD activity or changing prey capture ability. Two control groups (non-injected and injected with triolein only) were also included. Embryos were exposed, incubated and monitored as described in section 1.4.3. At 16 DPF, larvae were stored at -80°C until analyses. For each of these four treatments, three pools of five larvae were kept for EROD activity measurement. EROD activity was measured the same way as

described in section 1.4.6.

Total RNA was extracted from nine larvae per treatment (three pools of three larvae) using the Absolutely RNA Miniprep kit (Agilent®, Mississauga, ON, Canada) according to the manufacturer's instructions. There was one additional step involving the addition of a phenol-chloroform-isoamyl alcohol mixture (25:24:1) to the samples to eliminate proteins and provide a better isolation of RNA. Complementary DNA (cDNA) was synthesized from previously extracted RNA using the AffinityScript Multiple Temperature cDNA Synthesis kit (Agilent®, Mississauga, ON, Canada) following the manufacturer's instructions. Complementary DNA was stored at -20°C until needed.

Specifically designed and literature-based primers for target genes were used in this study and are listed in table 4. *actb* (beta-actin) was used as a housekeeping gene for qPCR normalization. Several studies demonstrated that beta-actin expression is not affected by TCDD exposure in zebrafish (*Danio rerio*) embryos (Handley-Goldstone et al., 2005; McCurley and Callard, 2008). Three target genes are related to the AHR machinery: *ahr1*, *ahr2* and *cyp1a*. Three other target genes are involved in the defense against oxidative stress: *gstm*, *sod1* and *sod2*. The gene *nNOS* was targeted because nitric oxide (NO) signaling is involved in early larval neurogenesis and organogenesis (Bradley, 2011; Holmqvist et al., 2004; Poon et al., 2003; Wang et al., 2004). Finally, *p53* was targeted because it encodes for the p53 tumor suppressor protein, a key regulator of the expression of numerous pro-apoptotic genes (Chipuk and Green, 2006).

For genes *ahr1*, *sod*1 and *sod2*, primers were designed using mRNA sequences for *F. heteroclitus* provided by the GenBank® database (accession numbers AF024591, CV824930 and CV821820, respectively) and the Roche Probe Design® software (Boulogne-Billancourt, France). The *p53* gene mRNA sequence was not available on GenBank® database for *F. heteroclitus*. Thus, mRNA sequences for *p53* and for the zebrafish (*Danio rerio*), the Japanese medaka (*Oryzias latipes*) and the Atlantic salmon (*Salmo salar*) were compared (accession numbers U60804, AF003950 and BT058777) and primers were designed to target mRNA

Table 4: Target genes and *F. heteroclitus* specific primers used in this study.

Gene name	Common name	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
actb	Beta-actin	CCATTGGCAACGAGAGGTTCC	CTCATCGTACTCCTGCTTGCTGATCC	Roling et al. (2004)
ahr1	Aryl hydrocarbon receptor 1	GGATTCTGAGCGTGTGCC	CTGCCCCACCAGATGAGG	This study
ahr2	Aryl hydrocarbon receptor 2	GCAGTGATGTACAACCCTGAGC	CCCGTGGAACTTCAGTGCCAGG	Aluru et al. (2011)
cyp1a	Cytochrome P4501A	TGTTGCCAATGTGATCTGTG	CGGATGTTGTCCTTGTCAAA	McElroy et al. (2012)
gstm	Glutathione *S*-transferase class Mu	TATGTGCGGAGAGACTGAGG	TCACAAAGCCGTTTCTGAAG	Roling et al. (2006)
nNOS	Neuronal nitric oxide synthase	GGAGACTGGGTGTGGATTGTG	CCGTTGCCAAATGTACTG	Hyndman et al. (2006)
p53	Tumor suppressor protein p53	TGGCGGACGTGGTTAAGAG	GCAGGAGCTGTTGCACAT	This study
sod1	Superoxide dismutase, cytosolic form (CuZn-SOD)	CTCAAGCTCTCAGGACCC	GGGTACTTTAGTGCGCCAT	This study
sod2	Superoxide dismutase, mitochondrial form (Mn-SOD)	GGCTGGGGATGGCTTG	ATCGTAGGCGTCGTGC	This study

regions well conserved among these three species. This primer pair was used to amplify *p53* in *F. heteroclitus*. The PCR product was cloned into the pGEM®-T (Promega, Charbonnières, France) plasmid and then sequenced. From this sequence (accession number KC171021) specific primer pair usable in qPCR reaction was determined as described above. All others primers sequences were obtained from the literature (table 4).

The qPCR reactions were performed with a MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad®, Mississauga, ON, Canada) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent®, Mississauga, ON, Canada) following the manufacturer's instructions. The amplification program consisted of one step of 10 min at 95°C (*Taq* polymerase hot-start) followed by 40 successive amplification cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. At the end of the run, melting curves were obtained by following the SYBR® Green fluorescence level with increasing temperature from 60°C to 95°C and were used to determine the specificity of each reaction.

Quantification of the level of expression of each target gene was normalized to the expression of the housekeeping gene *actb*. Relative expression of a given gene was calculated using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001), where ΔCt is defined as the difference between the cycle threshold of the target gene and the housekeeping gene beta-actin. Thus, the expression factor (EF) of a specific gene compared to control can be calculated from the following equation, where the treatment represents TCDD or PCB126 samples: $EF = \frac{2^{-\Delta Ct}(\text{treatment})}{2^{-\Delta Ct}(\text{injected control})}$.

1.4.8 Statistical analyses

Statistical analyses were conducted using SAS® 9.2 (SAS Institute Inc., Cary, NC, USA), with the significant level set at $\alpha = 0.05$. Normality of data before and after log-transformation was assessed with the Shapiro-Wilk test. Only log-transformed data for EROD activity were normally distributed. In this case, comparisons among treatments were per-

formed with a one-way ANOVA followed by a Tukey's multiple comparisons test. Other data were not normally distributed, thus comparisons among treatments were performed with a non-parametric Kruskal-Wallis test (KW). When significant differences were detected, it was followed by a multiple comparisons Tukey's studentized range test on the ranked values, as suggested by Conover and Iman (1981). When data were expressed as proportions of individuals (e.g. for mortality, hatching success and malformations), data were compared among treatment using the Fisher's exact test (FE).

Dose-response relationships for EROD activity, body length and prey capture ability were examined by least square regression. The homoscedasticity and the normal distribution (Shapiro-Wilk statistic) of residuals were assessed prior to regression analyses. Data for all parameters were expressed as percentages relative to triolein controls and log-transformed prior to regression analysis.

For EROD activity, after confirmation of the homogeneity of slopes by using the general linear models procedure of SAS®, the intercepts of the dose-response relationships of TCDD and PCB126 were compared using an analysis of covariance (ANCOVA). The relative potency of PCB126 to TCDD for EROD induction was estimated by comparing the dose of each compound inducing an effect equal to the upper 95% confidence limit of control larvae for this parameter (Parrott et al., 1995). These doses were calculated using regression lines, and the relative potency was defined as $\mathrm{ReP} = \frac{\mathrm{Dose}_{\mathrm{TCDD}}}{\mathrm{Dose}_{\mathrm{PCB126}}}$.

Correlations among the different responses (EROD activity, body length and prey capture ability) were assessed using the Spearman's rank correlation coefficient (ρ) test. Additionally, the influence of the presence or absence of craniofacial deformities (fig. 9) on body length, basal locomotor activity, reaction to a visual stimulus and prey capture ability and efficiency was tested. For doses of TCDD or PCB126 showing a significant increase of the prevalence of craniofacial deformities and taken individually, these responses were compared between impacted and non-impacted individuals (fig. 9) within doses using the Mann-Whitney U test (MW).

1.5 Results

1.5.1 Chemical analyses

Measured concentrations (17.08 ng μL^{-1} and 0.91 μg μL^{-1}) were very close to nominal concentrations (15 ng μL^{-1} and 1 μg μL^{-1}) for TCDD and PCB126 stock solutions, respectively. Similarly, the measured concentration of PCB126 in the working solution was 10.2 ng μL^{-1} compared to 12.5 ng μL^{-1} (nominal). Finally, concentrations of PCBs, PCDDs and PCDFs in pure triolein were below or very close to the detection limits (0.08 ng μL^{-1} for PCBs and approximately 0.05 pg μL^{-1} for PCDDs and PCDFs).

1.5.2 Mortality and hatching success

Triolein injection had no significant effect on mortality rate at 16 DPF, with 11% (0-22) and 6% (0-14) mortality in non-injected controls and 20% (7-33) and 9% (0-18) for injected controls for experiments with TCDD and PCB126, respectively (% mortality and 95% confidence interval). The cause of death of embryo during development in control groups was not precisely identified but could include poor quality of certain eggs at spawning, mechanical injury during manipulation or injection. Mortality was significantly increased only at the highest TCDD dose tested (1280 pg g^{-1} ww), with 37% (21-53) mortality at 16 DPF (FE, $p \leq 0.05$, data not shown). Treatment with PCB126 did not induce a significant increase in mortality.

There was no significant effect of triolein injection on hatching rate at 16 DPF, with hatching rates ranging from 90 to 100% in injected and non-injected controls from both experiments. Hatching rate was not affected by TCDD exposure at any doses. With PCB126, a slight reduction (FE, $p \leq 0.05$, data not shown) in hatching success was observed for the intermediate dose 1250 pg g^{-1} ww at 14 DPF, but not with the highest doses tested (2500 and 5000 pg g^{-1} ww). However, hatching success was no more impacted by PCB126 at 15 and 16

DPF.

1.5.3 Deformities and body length

The prevalence of craniofacial malformations was significantly increased at the two highest doses of PCB126, i.e. 2500 and 5000 pg g^{-1} ww, with respectively 22% (8-36) and 68% (51-85) of larvae affected (% affected and 95% confidence interval, FE, $p \leq 0.05$). Both upper and lower jaws were atrophied in the most affected larvae (fig. 9). A small proportion of larvae (≤9%) exposed to the highest doses of TCDD and PCB126 exhibited other abnormalities (edemas or hemorrhages, data not shown). Craniofacial deformities, edemas or hemorrhages were not observed in any control larvae.

TCDD and PCB126 caused a reduction in body length, which was statistically significant at 1280 and at ≥2500 pg g^{-1} ww, respectively (KW, $p \leq 0.05$) (fig. 10). At the highest doses of TCDD and PCB126 tested, the maximum effect observed was a 5% (3-7) and a 7% (4-10) reduction in body length, respectively (median value, Q1-Q3). No significant differences in body length were observed between non-injected and triolein injected control groups in both experiments (data not shown). The assumptions required (homoscedasticity, normality of residuals, etc.) were not met when performing linear regressions with body length data, thus dose-response relationships were not compared between TCDD and PCB126 for this parameter.

1.5.4 Locomotor activity

Exposure to TCDD or PCB126 had no effects on rate of travel, active swimming speed or inactivity during light or dark periods (data not shown). Recovery latency and duration of resting times during the light period were not significantly altered by TCDD. The number of resting periods tended to increase with the dose of TCDD, but it was not statistically significant ($R^2 = 0.07$, $p = 0.0925$). At the highest dose (5000 pg g^{-1} ww), PCB126 tended to

increase the recovery latency period and the duration of the resting times and to decrease the number of resting times, during the light period. However, these trends were not significant with high inter-individual variability (table 5). Intravitelline injection of pure triolein had no effect on basal locomotor activity or reaction to light compared to non-injected controls (table 5).

1.5.5 Prey capture ability

The number of *Artemia* remaining after 5 min of predation was significantly higher at 1280 pg g^{-1} ww for TCDD and at ≥2500 pg g^{-1} ww for PCB126 (KW, $p \leq 0.05$) (fig. 10). The rate of decline of *Artemia* in the well (i.e. the slope of the relationship between the number of *Artemia* and time, logarithmically transformed) was also significantly reduced at the same doses of TCDD or PCB126 (KW, $p \leq 0.05$) (table 5). Total number of feeding strikes was not affected by any treatment. However, prey capture efficiency (i.e. number of *Artemia* captured per feeding strike) was significantly reduced (KW, $p \leq 0.05$) at ≥320 pg g^{-1} ww for TCDD and at 5000 pg g^{-1} ww for PCB126 (table 5). The relationships between the number of *Artemia* captured and the number of feeding strikes showed that some larvae exposed to either TCDD (1280 pg g^{-1} ww) or PCB126 (5000 pg g^{-1} ww) captured relatively few preys despite attempting a large number of feeding strikes (fig. 11).

Both TCDD (R^2 = 0.19, $p \leq 0.05$, F = 8.34, DF = 35) and PCB126 (R^2 = 0.75, $p \leq 0.05$, F = 103.94, DF = 35) reduced prey capture ability of larvae in a dose-dependent manner. Slopes of the dose-response relationships were not homogeneous ($p \leq 0.001$), thus difference between intercepts was not assessed. The slope of the dose-response relationship for prey capture ability was steeper for PCB126 (0.78 ± 0.08) compared to TCDD (0.19 ± 0.07).

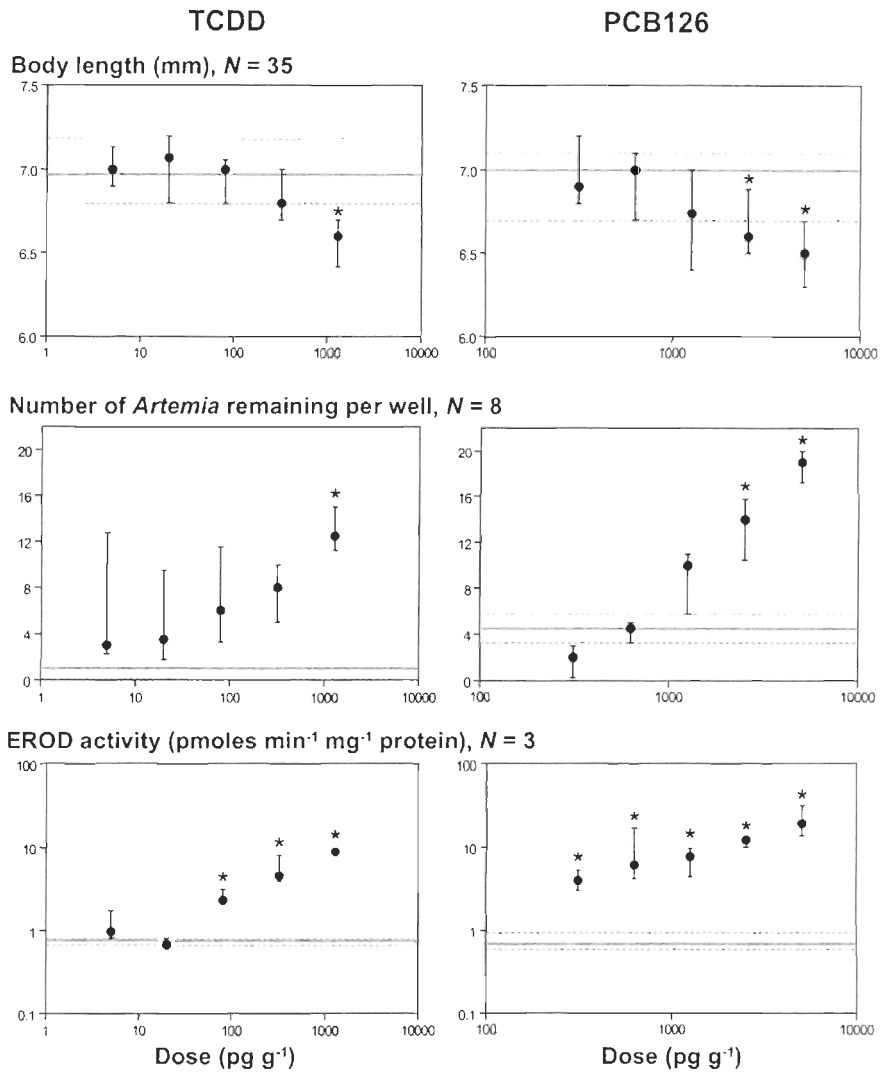

Figure 10: Effect of TCDD and PCB126 on body length, prey capture ability (expressed here as the number of *Artemia* remaining per well after 5 min of predation) and EROD activity (log-transformed) of *F. heteroclitus* larvae at 16 DPF. Values are expressed as median (dots) and interquartile range (vertical bars). Solid and dotted grey lines represent median value and interquartile range for the triolein control group, respectively. The non-injected (Ni) control group is not shown. * indicates a significant difference compared to the triolein control group (ANOVA or KW, $p \leq 0.05$, see section 1.4.8 for details).

Table 5: Effect of TCDD and PCB126 on the reaction to a light stimulus and the predation of *F. heteroclitus* larvae at 16 DPF. Values are expressed as medians (first and third quartiles in parenthesis). The non-injected (Ni) control group was not assessed for the predation assay. $N = 8$ per dose.

	Reaction to a light stimulus[a]			Predation[b]		
Dose of TCDD ($pg\ g^{-1}$)	Recovery latency period (s)	Number of resting periods	Duration of resting periods (s)	Prey capture ability[c]	Number of feeding strikes	Prey capture efficiency[d]
Ni	3.5 (1.5 10.3)	18.5 (13.8 23.0)	3.6 (2.6 5.5)	-	-	-
0	11.5 (5.3 25.0)	21.5 (12.5 29.3)	4.8 (3.8 5.1)	-5.0 (-5.4 -4.1)	24.0 (21.5 27.3)	0.7 (0.6 0.9)
5	4.0 (2.0 7.3)	23.5 (16.5 27.5)	3.7 (3.4 4.1)	-4.6 (-4.9 -3.5)	23.5 (19.3 25.5)	0.6 (0.6 0.7)
20	4.0 (1.5 11.0)	27.5 (15.5 36.0)	4.2 (3.8 5.0)	-4.3 (-4.5 -4.0)	23.5 (20.5 27.3)	0.7 (0.6 0.7)
80	7.5 (3.0 20.8)	26.0 (19.5 27.3)	3.3 (3.0 6.2)	-3.8 (-4.6 -2.7)	22.0 (18.0 27.3)	0.6 (0.5 0.7)
320	8.5 (5.0 25.3)	30.0 (29.0 34.3)	4.4 (3.7 5.2)	-3.9 (-3.9 -3.0)	24.0 (23.0 28.5)	0.5 (0.5 0.6) *
1280	5.5 (3.5 8.3)	31.0 (26.8 33.5)	5.2 (4.5 6.1)	-1.8 (-2.4 -1.4) *	23.5 (18.5 30.5)	0.4 (0.2 0.4) *
Dose of PCB126 ($pg\ g^{-1}$)						
Ni	8.0 (3.8 13.8)	33.5 (28.3 35.0)	4.5 (3.6 6.2)	-	-	-
0	4.50 (3.8 6.8)	29.0 (26.5 31.0)	4.4 (3.6 6.1)	-4.0 (-4.5 -3.3)	24.5 (21.3 27.5)	0.7 (0.5 0.7)
312	16.0 (7.3 26.0)	24.5 (22.0 30.0)	4.7 (4.2 5.9)	-4.6 (-4.9 -4.3)	23.5 (20.8 29.0)	0.8 (0.6 0.8)
626	10.5 (7.0 15.3)	30.5 (26.5 34.8)	4.9 (4.2 6.4)	-4.1 (-4.3 -4.0)	23.5 (21.8 31.8)	0.6 (0.5 0.7)
1250	4.0 (3.0 15.3)	31.0 (29.8 37.0)	4.2 (4.0 4.8)	-2.6 (-3.5 -2.3)	17.5 (14.0 22.8)	0.6 (0.4 0.7)
2500	4.5 (3.8 12.0)	28.0 (23.0 32.6)	6.4 (4.4 8.6)	-1.9 (-2.6 -1.4) *	19.5 (15.8 24.0)	0.4 (0.4 0.4)
5000	51.5 (12.5 76.0)	20.5 (14.3 22.5)	9.8 (6.2 19.4)	-0.4 (-0.7 -0.2) *	16.5 (13.3 23.3)	0.1 (0.0 0.2) *

[a] Parameters are measured during the 5 minutes of recording under white light condition with the Zebrabox® system (see section 1.4.4 for details).

[b] Parameters are measured during the 5 minutes of predation assay (see section 1.4.5 for details).

[c] Prey capture ability is expressed as the rate of decline of *Artemia* in the well, i.e. the slope of the relationship between the number of *Artemia* remaining in the well and time (logarithmically transformed).

[d] Prey capture efficiency is expressed as the number of *Artemia* captured per feeding strike.

* indicates significant difference compared to respective (TCDD or PCB126) control group (KW, $p \leq 0.05$).

1.5.6 EROD activity

EROD activity increased with dose for both TCDD (R^2 = 0.92, $p \leq 0.05$, F = 102.72, DF = 9) and PCB126 (R^2 = 0.81, $p \leq 0.05$, F = 51.83, DF = 12), with significant induction at ≥80 and at ≥312 pg g^{-1} ww, respectively (fig. 10). The lowest dose of TCDD (5 pg g^{-1}) was excluded for the calculation of the dose-response relationships because it produced no significant effect and reduced the R^2. Comparison of the dose-response relationships of TCDD and PCB126 for EROD activity revealed that slopes were homogeneous (p = 0.80) and that intercepts were similar (ANCOVA, p = 0.22). The upper 95% confidence limit for EROD activity of control larvae was equal to 0.82 pmoles resorufin min^{-1} mg^{-1} protein. Corresponding doses of TCDD and PCB126 were estimated from regression lines at 19.5 and 27.3 pg g^{-1} ww, respectively. Thus, the relative potency of PCB126 versus TCDD to EROD induction was 0.71. EROD activity was not induced by injection of triolein alone (data not shown).

1.5.7 Genes expression levels

EROD activity was similar and significantly induced (compared to controls) in larvae used for qPCR analyses and exposed to 1280 pg g^{-1} ww of TCDD or 1250 pg g^{-1} ww of PCB126 (table 6). Data for gene expression levels are shown in table 6. The *cyp1a* gene was the most responsive with a significant 45- and 54-fold up-regulation in TCDD and PCB126 exposed larvae, respectively, without any significant difference between these two treatments. The *sod2* gene was significantly 3-fold up-regulated following exposure to PCB126, with no changes for this gene in larvae exposed to TCDD. The *p53* gene tended to be up-regulated in the TCDD treatment, however this was not significant. Similarly, *ahr1*, *ahr2* and *nNOS* tended to be up-regulated in the PCB126 treatment, without any significant trend. Gene expression levels did not differ significantly between non-injected and injected controls, except for *ahr2* and *nNOS* where expression levels tended to be lower in non-injected controls. The *gstm* and *sod1* genes were at the same level of expression for all treatments.

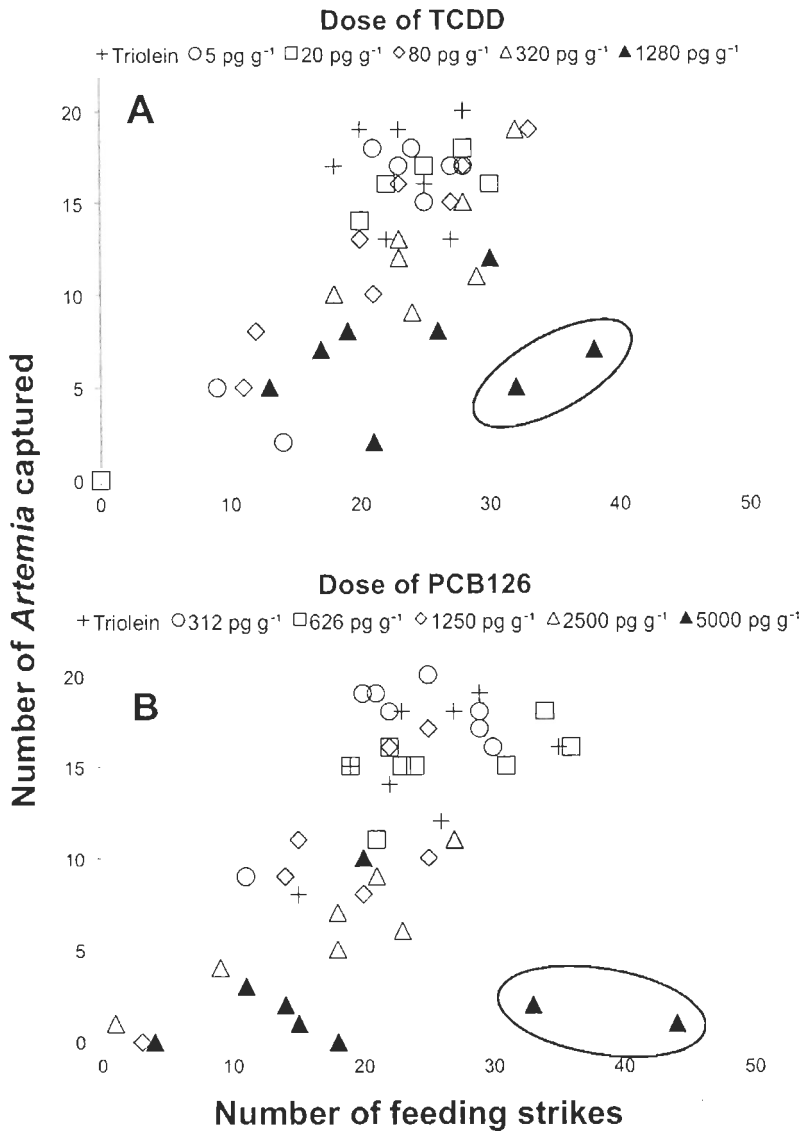

Figure 11: Relationship between the number of *Artemia* captured and the number of feeding strikes for each larva, following treatment with TCDD (A) or PCB126 (B). Ellipses circle some larvae capturing a small number of prey despite attempting a large number of feeding strikes. $N = 8$ per dose.

Table 6: EROD activity (expressed as pmol min^{-1} mg^{-1} protein) for *F. heteroclitus* larvae (16 DPF) used in qPCR analysis and expression factors of the target genes comparatively to their basal level in injected controls (N = 3 pools of 5 larvae for EROD activity and 3 pools of 3 larvae for qPCR analysis).

Treatment	EROD activity	Expression factors							
		ahr1	*ahr2*	*cyp1a*	*gstm*	*nNOS*	*p53*	*sod1*	*sod2*
Ni	0.70 ± 0.16	0.81 ± 0.19	0.35 ± 0.12	1.80 ± 0.98	0.91 ± 0.06	0.48 ± 0.11	1.06 ± 0.44	1.00 ± 0.19	0.53 ± 0.35
Triolein	0.89 ± 0.18	1.00 ± 0.54	1.00 ± 0.43	1.00 ± 0.16	1.00 ± 0.26	1.00 ± 0.33	1.00 ± 0.13	1.00 ± 0.22	1.00 ± 0.62
TCDD	3.56 ± 0.57 *	1.12 ± 0.05	1.31 ± 0.97	45.09 ± 17.92 *	1.05 ± 0.55	1.45 ± 1.11	2.17 ± 0.72	1.13 ± 0.91	1.48 ± 0.77
PCB126	4.06 ± 1.39 *	1.77 ± 0.65	1.98 ± 1.09	53.68 ± 26.99 *	0.88 ± 0.33	2.32 ± 0.80	1.12 ± 0.40	1.03 ± 0.06	3.12 ± 1.16 *

Note: Ni refers to the non-injected control group, Triolein refers to the injected control group. TCDD and PCB126 doses were respectively 1280 and 1250 pg g^{-1}.

* indicates a significant p-value compared to the injected control group (ANOVA or KW, $p \leq 0.05$, see section 1.4.8 for details).

1.5.8 Correlation between different responses

For both compounds, EROD activity was strongly correlated to prey capture ability, prey capture efficiency and body length (ρ = 0.90, -1.00 and -0.97 for TCDD, and ρ = 1.00, -0.89 and -0.90 for PCB126, respectively). For both compounds, body length was significantly and negatively correlated to prey capture ability (ρ = -0.50 and -0.65 for TCDD and PCB126, respectively) as well as significantly and positively correlated to prey capture efficiency (ρ = 0.64 and 0.62 for TCDD and PCB126, respectively).

The influence of the presence or absence of craniofacial deformities was studied for the two highest doses of PCB126 (2500 and 5000 pg g^{-1} ww) where the prevalence of craniofacial deformities was significantly increased (see section 1.5.3). At 5000 pg g^{-1} ww, individuals with a craniofacial deformity were significantly smaller compared to unaffected individuals (MW, $p \leq 0.05$, N = 28), but not at 2500 pg g^{-1} ww (MW, $p > 0.05$, N = 30). Among individuals used for the behavioral tests (basal locomotor activity and prey capture capabilities), seven of the eight larvae exposed to 5000 pg g^{-1} ww had a craniofacial deformity, thus the relationships between the prevalence of craniofacial deformities and prey capture capabilities could not be examined. At 2500 pg g^{-1} ww, no significant effect of the presence or absence of craniofacial deformities on prey capture capabilities, basal locomotor activity or reaction to a visual stimulus was observed (MW, $p > 0.05$, N = 8). However, the number of *Artemia* captured after 5 min of predation as well as the rate of decline of *Artemia* during this period tended to be slightly lower for larvae with a craniofacial deformity.

1.6 Discussion

1.6.1 Relative potency of PCB126 versus TCDD to induce EROD activity

Relative potency of PCB126 compared to TCDD was assessed for EROD activity, which was the most sensitive endpoint. This study demonstrates a markedly higher PCB126

ReP for *F. heteroclitus* compared to rainbow and lake trout (tables 7 and 8). To our knowledge, this study provides the highest ReP (0.71) ever reported for PCB126 in fish, approximately 140-fold higher than the WHO TEF of PCB126 (0.005) derived from *in ovo* embryolethality tests in trout species (Van den Berg et al., 1998). The ReP of PCB126 to TCDD based on EROD induction for mummichog embryo (0.71) is 500 times higher compared to the ReP for EROD induction for juvenile rainbow trout (0.0014) (table 7). Very few studies report PCB126 RePs for non-salmonids species. RePs derived from *in vitro* studies on CYP1A induction in zebrafish and clearfin livebearer (*Poeciliopsis lucida*) hepatoma cells and for embryolethality in Japanese medaka are within the range of those reported for trout (tables 7 and 8). The high ReP value (0.35) reported by Hahn et al. (1996) for EROD activity in clearfin livebearer cells is, as stated by the authors, a consequence of EROD inhibition at high doses of PCB126 which is not observed in our study. There was an attempt to determine an *in vivo* ReP for hepatic EROD induction in the common carp (*Cyprinus carpio*) (van der Weiden et al., 1994) which failed as a result of improper dose selection but suggested that the TEF of 0.005 underestimated PCB126 toxicity in this species.

High interspecies variability for *in vivo* and *in vitro* PCB126 RePs (0.000067 to 0.86) has been reported in mammals (Haws et al., 2006). This high variability was largely attributable to the inclusion of data from *in vitro* studies conducted on human cells whose geometric mean ReP were 0.007 compared to 0.1 for nonhuman cell studies (Haws et al., 2006; Sutter et al., 2010). The exact cause of this variability is not known but differences in ligand-specific AHR-coactivator interactions could be involved (Carlson et al., 2009; Zhang et al., 2008). Fish species display a large variability in the number, type and expression pattern of AHR pathway genes (Zhou et al., 2010) which can contribute to interspecies variation in RePs.

Table 7: Overview of some relative potencies of PCB126 versus TCDD determined from *in vivo* studies and for AHR-related biochemical endpoints and mortality in fish.

Endpoint and species	Tissues	Route of exposure	Relative potency	Reference
AHH[a]				
Oncorhynchus mykiss	Liver (adults)	IP[b] injection (corn oil)	0.005	Janz and Metcalfe (1991)
	Liver (juveniles)		0.054	Newsted et al. (1995)
EROD				
Cyprinus carpio	Liver	IP[b] injection (peanut oil)	> 0.005[c]	van der Weiden et al. (1994)
Fundulus heteroclitus	**Larvae**	**IV[d] injection (triolein)**	**0.71**	**This study**
Oncorhynchus mykiss	Liver (juveniles)	IP[b] injection (corn oil)	0.0014	Newsted et al. (1995)
Mortality				
Oncorhynchus mykiss	Embryos/Larvae	IV[d] injection (liposomes)	0.005 (0.003-0.01)	Walker and Peterson (1991)
Oryzias latipes		Aqueous (acetone)	0.032	Kim and Cooper (1999)
Salvelinus namaycush			0.003[e] (0.0011-0.012)	Zabel et al. (1995a)

Note: All RePs listed here were estimated by their respective authors using ED_{50}s values.

[a] Aryl hydrocarbon hydroxylase.

[b] Intraperitoneal.

[c] Based on internal dose measured in liver.

[d] Intravitelline.

[e] Based on internal dose measured in whole embryos.

Table 8: Overview of some relative potencies of PCB126 versus TCDD determined from *in vitro* studies and for AHR-related biochemical endpoints in fish.

Endpoint and species	Tissues	Relative potency	Reference
CYP1A mRNA			
Danio rerio	Liver cells (ZF-L)	0.01 ± 0.002	Henry et al. (2001)
Oncorhynchus mykiss	Gonadal cells (RTG-2)	0.056	Zabel et al. (1996)
CYP1A protein			
Poeciliopsis lucida	Hepatoma cells (PLHC-1)	0.027	Hahn et al. (1996)
EROD			
Oncorhynchus mykiss	Gill epithelial cells	0.0035 ± 0.0026	Carlsson et al. (1999)
		0.007	Carlsson and Pärt (2001)
	Liver cells (RTL-W1)	0.023 ± 0.0046	Clemons et al. (1996, 1997)
	Pituitary cells (RTP-2)	0.037	Tom et al. (2001)
	Pituitary cells (RTP-91E)	0.033	
	Pituitary cells (RTP-91F)	0.055	
Poeciliopsis lucida	Hepatoma cells (PLHC-1)	0.24[a]	Hahn and Chandran (1996)
		0.35	Hahn et al. (1996)

Note: All RePs listed here were estimated by their respective authors using ED_{50}s values. Route of exposure for all studies listed in this table was aqueous with DMSO as a solvent.

[a] In this study, the relative potency of PCB126 versus TCDD was not directly provided by the authors, but was estimated from ED_{50}s values.

1.6.2 Effect of TCDD and PCB126 on behavior of mummichog larvae

This study shows that both TCDD and PCB126 caused a dose-responsive reduction of prey capture ability (fig. 10). PCB126 seems at least as potent as TCDD to reduce prey capture ability of *F. heteroclitus* larvae. However, a ReP could not be calculated for this endpoint due to different slopes. To our knowledge, no studies have compared the ReP of DLCs to induce neurotoxic or behavioral alterations in fish. Even in mammals, only one paper has compared the ReP of PCB126 and TCDD for behavioral endpoints: Hojo et al. (2008) demonstrated that the WHO TEF of PCB126 for mammals (0.1) corresponded closely to the ReP for effects on learning behavior of rat pups following maternal exposure to PCB126 and TCDD. In the present work, at TCDD-equivalent low and medium doses, TCDD and PCB126 had similar effects, while at a high dose PCB126 was more potent, suggesting a different mechanism of action.

Following exposure to PCB126, reduced predatory capacities were observed only with two doses (2500 and 5000 pg g^{-1} ww) which also caused an increase of the prevalence of craniofacial deformities. At 2500 pg g^{-1} ww of PCB126, no significant effect of the presence of craniofacial deformities on prey capture capabilities was observed. However, this effect was not testable at 5000 pg g^{-1} ww of PCB126 due to the virtual absence of non-deformed larvae at this dose (only one of eight larvae involved in behavioral tests was concerned). Thus, the influence of a shortened snout on reduced prey capture ability of larvae exposed to high doses of PCB126 cannot be ruled out. However, at the lowest PCB126 doses (e.g. 1250 pg g^{-1} ww) or following exposure to TCDD (≤1280 pg g^{-1} ww), the prey capture capabilities of several larvae were altered without any craniofacial deformities (figs. 10 and 11). For both compounds, prey capture ability was reduced without any detectable alteration of larvae basal locomotor activity. Furthermore, some larvae exposed to the highest doses of TCDD (1280 pg g^{-1} ww) or PCB126 (5000 pg g^{-1} ww) captured relatively few prey despite attempting a large number of feeding strikes (fig. 11). A similar pattern was observed previously by Couillard et al. (2011) with *F. heteroclitus* larvae exposed topically to sublethal

doses of PCB126 (up to 50 pg egg^{-1}). In the present study, prey capture capabilities of larvae were also negatively correlated with body length, suggesting retarded growth as a possible cause for altered behavior. However, Couillard et al. (2011) found that retarded growth of *F. heteroclitus* was also associated with a reduced number of feeding strikes in some cases, which was not observed in the present study for any TCDD or PCB126-exposed larvae.

Taken together, these previous observations suggest that prey capture ability may be reduced by mechanisms other than jaw deformity, retarded growth or reduced motility. TCDD reduced prey capture ability and the density of retinal ganglion cells in TCDD-injected (≥300 pg g^{-1} ww) rainbow trout larvae, suggesting impaired vision as a possible mechanism for reduced predatory capacity (Carvalho and Tillitt, 2004). In the present paper, the results are consistent with the impaired vision hypothesis, but other mechanisms should be considered such as delayed fetal brain development (Nishijo et al., 2007) or decreased learning abilities (Piedrafita et al., 2008) that have been demonstrated in rodents and not yet extensively investigated in fish.

1.6.3 Effects of TCDD and PCB126 on gene expression

Measured AHR-related responses (EROD activity and *cyp1a* mRNA levels) suggest that gene expression levels were studied in larvae exposed to similar TCDD-equivalent doses of TCDD or PCB126 (table 6). Except *cyp1a*, only *sod2* mRNA levels were significantly altered by exposure to PCB126 but not by TCDD (table 6). Consistent with our results and at similar *cyp1a* mRNA levels (50-fold induction compared to controls), Wills et al. (2010) showed a moderate and non-significant induction of *ahr2* mRNA levels (1.5 fold compared to controls) in *F. heteroclitus* embryos (6 DPF) waterborne exposed to PCB126 (1 μg L^{-1}). However, *ahr1* was not studied by these authors. Exposure to either TCDD or PCB126 was reported to cause a moderate induction (2- to 4-fold) of *ahr1* or *ahr2* mRNA levels in zebrafish cells culture (Tanguay et al., 1999) or in embryos of the zebrafish or the red seabream (*Pagrus major*) (Andreasen et al., 2002; Handley-Goldstone et al., 2005; Yamauchi

et al., 2006), at doses causing severe deformities related to the BSD syndrome. Consistent with our results, Powell et al. (2000) did not observe any significant induction of *ahr1* or *ahr2* mRNA levels following aqueous exposure of mummichog larvae to two concentrations of TCDD (2 and 20 nM). These authors have not mentioned any results regarding deformities. Tanguay et al. (1999) observed a concentration-dependent induction of *ahr2* in zebrafish embryos exposed to TCDD at concentrations (up to 30 nM) causing BSD syndrome in this species. Thus, it is possible that high doses causing severe deformities are more likely to induce *ahr1* or *ahr2* genes and/or that responses of AHR genes differ among species.

The level of expression of several genes involved in the mechanisms of defense against oxidative stress was also measured: *gstm*, *sod1* and *sod2* (tables 4 and 6). Levels of *gstm* or *sod1* mRNA were not significantly altered by exposure to TCDD or PCB126 (respectively 1280 and 1250 pg g^{-1} ww). However, there was a significant 3-fold induction of the expression of *sod2* following exposure to PCB126, with no apparent effect of TCDD on this gene. Coplanar PCBs are known to induce oxidative stress in mammals (Hennig et al., 2002), birds (Jin et al., 2001) and fish (Schlezinger et al., 2006), possibly by uncoupling the cytochrome P4501A cycle and increasing the release of reactive oxygen species (ROS). Because ROS are generated mainly in the mitochondria, *sod2*, which encodes Mn-SOD (the mitochondrial form of the superoxide dismutase enzyme), is thought to be a more physiologically important antioxidant than *sod1* (Erker et al., 2006). In zebrafish embryos exposed to waterborne PCB126 (100 nM or approximately 32.6 μg μL^{-1}), the protective effect of the anti-oxidant vitamin E against PCB126 embryotoxicity was associated with an increase of the expression of *sod2* mRNA (Na et al., 2009). Exposure to PCB126 alone (without vitamin E) also induced *sod1* mRNA in zebrafish embryos (Na et al., 2009), while we did not observe any induction of this gene. Difference in species or in PCB126 dose may explain these differences. The unique dose of PCB126 (waterborne exposure, 100 nM or approximately 32.6 μg μL^{-1}) used by Na et al. (2009) was obviously higher compared to the dose we used here (1250 pg g^{-1} ww): these authors observed severe deformities (yolk sac and pericardial edemas) not observed in our study with *F. heteroclitus* larvae exposed to 1250 pg g^{-1} ww.

To summarize, at comparable doses producing similar AHR-related responses, a different pattern was observed on the transcriptional response of studied genes between TCDD and PCB126. PCB126 appeared to enhance antioxidant responses by inducing *sod2* expression, while TCDD did not. It suggests that these two compounds act through different mechanisms to produce embryotoxicity or behavioral alterations in *F. heteroclitus* larvae, and that oxidative stress may be a key factor in the case of PCB126. However, due to experimental constraints and the limited availability of *F. heteroclitus* genome resources, we have studied only a reduced number of genes, which are not representative of all potential targets of TCDD and PCB126. When the *F. heteroclitus* genome is fully sequenced, large-scale analysis of gene expression such as interactomes may be useful to explore embryotoxic mechanisms of DLCs, as recently done in zebrafish embryos exposed to TCDD (Alexeyenko et al., 2010). Because the qPCR analyses were performed in pools of whole larvae and were not tissue-specific, it is also possible that the transcriptomic signal of some genes was too diluted to be measured as a significant response (e.g. for *nNOS* or *p53*). Techniques such as the whole-mount *in situ* hybridization are designed to study the spatial pattern of gene expression and have already been successfully used in *F. heteroclitus* embryos (Dong et al., 2008). These techniques lack the quantitative advantage of the PCR but may be useful to complete the results shown here and to explore the association between gene expression in the brain and behavioral responses.

1.6.4 Relevance of the study

Concentrations of TCDD in lake trout females or eggs of Lake Ontario during the late 1980s were measured by Cook et al. (2003) at 33.5 and 11.2 pg g^{-1} ww, respectively. Nowadays, concentrations of TCDD have declined in lake trout from Lake Ontario and are below 10 pg g^{-1} ww (Bhavsar et al., 2008a). This study showed deleterious effects of TCDD on prey capture ability at doses well above the concentrations in lake trout. However, the highest doses of PCB126 used in this study were similar to measured concentrations of PCB126 in lake trout females or eggs in the late 1980s (respectively 2470 and 731 pg g^{-1} ww, close

to the range of doses of PCB126 tested in this study) by Cook et al. (2003). Thus, the results presented here suggest that environmentally relevant concentrations of PCB126 could affect the prey capture ability of sensitive fish species. Behavioral responses are generally sensitive to low levels of a variety of environmental contaminants and can have major ecological significance by altering fitness, migratory competence and recruitment success (Weis and Candelmo, 2012). Further studies are needed to assess the relative potencies of other DLCs to alter prey capture ability and to verify if their toxicities are additive. This study is also the first to report that the WHO TEF for PCB126 in fish may underestimate the relative potency of this compound for sublethal effects at environmentally relevant doses. As previously observed in mammals (Haws et al., 2006; Sutter et al., 2010), this study demonstrates that major interspecies differences in terms of relative potency may also exist in fish. Thus, extreme care is needed when using WHO TEFs with non-salmonids species. To provide accurate risk assessments, we recommend the development of RePs specific to species and endpoints.

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ARTICLE II

APPLICABILITY OF THE TCDD-TEQ APPROACH TO PREDICT SUBLETHAL EMBRYOTOXICITY IN *FUNDULUS HETEROCLITUS*

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Contribution de chaque auteur - **C. Rigaud** : conception du projet, expérimentation et analyse des données, rédaction de l'article et conception des figures ; **C. M. Couillard** : conception du projet, supervision, aide à l'expérimentation et à la rédaction ; **J. Pellerin** : supervision et aide à la rédaction ; **B. Légaré** : aide à l'expérimentation ; **P. V. Hodson** : direction et coordination du projet CRSNG, supervision et aide à la rédaction.

2.1 Résumé

Le concept d'équivalent toxique en 2,3,7,8-tetrachlorodibenzo-*p*-dioxine (EQT-TCDD) a été utilisé avec succès pour prédire l'embryo-mortalité chez les salmonidés, mais son applicabilité aux effets sous-létaux de mélanges complexes de composés organohalogénés chez d'autres espèces de poissons est peu connue. La toxicité sous-létale de deux composés coplanaires, le 3,3',4,4'-tetrachlorobiphényle (BPC77) et le 2,3,4,7,8-pentachlorodibenzofurane (2,3,4,7,8-PnCDF), de deux biphényles polychlorés (BPC) non-coplanaires, le 2,2',5,5'-tetrachlorobiphényle (BPC52) et le 2,3,3',4',6-pentachlorobiphényle (BPC110), ainsi que d'un mélange comnplexe de BPC, l'Aroclor 1254, a été étudiée chez des embryons de *Fundulus heteroclitus* exposés par injection intravitelline. À 16 jours post-fécondation, les deux composés coplanaires et l'Aroclor 1254 ont altéré la capacité de prédation des larves en plus d'induire des réponses typiques liées au récepteur Ah : induction de l'activité de l'ethoxyrésorufine-*O*-dééthylase (EROD), malformations crânio-faciales et réduction de la taille des larves. Aucun de ces paramètres n'a été altéré par les deux BPC non-coplanaires à des doses allant jusqu'à 5400 ng g^{-1} de poids humide (p.h.). Les pentes des relations dose-réponse pour la capacité de prédation des deux composés coplanaires n'étaient pas parallèles à celle de la TCDD, violant un prérequis fondamental à l'estimation de leur potentiel toxique relatif (PTR). Les pentes des relations dose-réponse pour l'activité EROD étaient parallèles pour la TCDD et le 2,3,4,7,8-PnCDF, mais le PTR du 2,3,4,7,8-PnCDF pour *Fundulus heteroclitus* était 5 fois plus élevé que le facteur d'équivalence toxique (FET) de référence de l'Oganisation Mondiale de la Santé (OMS) pour les poissons, basé sur des données d'embryo-mortalité obtenues chez les salmonidés. Les EQT-TCDD dérivés des données chimiques et calculés à partir des concentrations en BPC126 et de son PTR chez *Fundulus heteroclitus* surestimaient la capacité de l'Aroclor 1264 à induire l'activité EROD, possiblement en raison d'interactions antagonistes entre les différents congénères de BPC. Cette étude met en évidence les limites de l'utilisation des FET basés uniquement sur des données obtenues chez les salmonidés pour l'évaluation des risques chez d'autres espèces de poissons. Il est

nécéssaire d'étudier la variabilité des PTR des composés coplanaires chez différentes espèces, pour une gamme de réponses variées, et de mieux comprendre les interactions entre les composés coplanaires et d'autres composés toxiques.

2.2 Abstract

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxic equivalent quantity (TCDD-TEQ) approach was used successfully to predict lethal embryotoxicity in salmonids, but its applicability to sublethal effects of mixtures of organohalogenated compounds in other fish species is poorly known. The sublethal toxicity of two dioxin-like compounds (DLCs), 3,3',4,4'-tetrachlorobiphenyl (PCB77) and 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PnCDF), two non-dioxin-like (NDL) polychlorinated biphenyls (PCBs), 2,2',5,5'-tetrachlorobiphenyl (PCB52) and 2,3,3',4',6-pentachlorobiphenyl (PCB110), and of Aroclor 1254, a complex commercial mixture of PCBs, was assessed in *Fundulus heteroclitus* embryos exposed by intravitelline injection. At 16 days post-fertilization, the two DLCs and Aroclor 1254 altered prey capture ability in addition to inducing classical aryl hydrocarbon receptor-mediated responses: ethoxyresorufin-*O*-deethylase (EROD) induction, craniofacial deformities and reduction in body length. None of these responses was induced by the two NDL PCBs, at doses up to 5400 ng g^{-1} wet weight. Dose-response curves for prey capture ability for the 2 DLCs tested were not parallel to that of TCDD, violating a fundamental assumption for relative potency (ReP) estimation. Dose-response curves for EROD induction were parallel for 2,3,4,7,8-PnCDF and TCDD, but the ReP of 2,3,4,7,8-PnCDF for *F. heteroclitus* was 5-fold higher than the World Health Organization (WHO) fish toxic equivalent factor (TEF) based on embryolethality in salmonids. The chemically derived TCDD-TEQs of Aroclor 1254, calculated using 3,3',4,4',5-pentachlorobiphenyl (PCB126) concentrations and it ReP for *F. heteroclitus*, overestimated its potency to induce EROD activity possibly due to antagonistic interactions among PCBs. This study highlights the limitations of using TEFs based on salmonid toxicity data alone for risk assessment to other fish species. There is

a need to assess the variability of RePs of DLCs in different species for a variety of endpoints and to better understand interactions between DLCs and other toxic chemicals.

2.3 Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs, 75 congeners), -furans (PCDFs, 135 congeners) and non-ortho-substituted (also called coplanar) polychlorinated biphenyls (PCBs, 4 congeners) are all planar halogenated hydrocarbons (PHHs). These dioxin-like compounds (DLCs) are persistent, widespread and lipophilic organic pollutants that bioaccumulate in marine organisms. PHHs interact with organisms through a cytosolic receptor, the aryl hydrocarbon receptor (AHR) (Poland and Glover, 1976). The affinity of PHHs for AHR binding is stronger when their structure is similar to that of the most toxic PHH, i.e. the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Safe, 1990). In fish early life stages, high doses of DLCs induce a syndrome called blue sac disease (BSD) characterized by induction of cytochrome P4501A (CYP1A) enzymes, developmental delay, craniofacial malformations, hemorrhages, pericardial and yolk sac edemas, responses that lead to death (Henry et al., 1997; Hill et al., 2004a,b; Teraoka et al., 2002). DLCs reached lethal concentrations in lake trout (*Salvelinus namaycush*) eggs in Lake Ontario (Canada) in the mid 20th century, and have declined since the beginning of the 1980s to sublethal concentrations (Cook et al., 2003). Sublethal doses of TCDD or of 3,3',4,4',5-pentachlorobiphenyl (PCB126) reduce prey capture ability in early life stages of the marine model fish *Fundulus heteroclitus*, the mummichog (Couillard et al., 2011; Rigaud et al., 2013), as previously demonstrated in rainbow trout (*Oncorhynchus mykiss*) exposed to TCDD (Carvalho and Tillitt, 2004). Impairment of the ability of fish larvae to capture prey could lead to reduced growth and survival with potential impacts on population recruitment (Weis and Candelmo, 2012).

Toxic equivalent factors (TEFs; i.e. the relative toxicity of each PHH versus TCDD, the reference PHH) were developed as tools for risk assessment to predict the toxic potency

of complex mixtures of PHHs from their TCDD toxic equivalent quantities (TCDD-TEQs, i.e. the sum of each PHH concentration times its TEF) (van Zorge et al., 1989). World Health Organization (WHO) reference TEFs for fish are based on embryolethality studies of trout species exposed to single PHHs by intravitelline (IVi) injection (Van den Berg et al., 1998). Expressing a complex mixtures of PHHs as an equivalent concentration of TCDD assumes that (1) all congeners in the mixture cause toxicity via a common mechanism and (2) that all congeners act additively to produce toxicity (Walker et al., 1996a). WHO fish TEFs and their additive model of toxicity were used successfully to investigate temporal changes in DLC-induced embryolethality in lake trout exposed to complex mixtures of organohalogenated compounds in Lake Ontario (Cook et al., 2003; Walker et al., 1996a; Wright and Tillitt, 1999). However, the applicability of this approach to other fish species and to sublethal responses is questionable: non-additive interactions have been reported for biochemical responses (CYP1A induction) in the rainbow trout and in the Japanese medaka (*Oryzias latipes*) exposed to simple binary mixtures of TCDD and a second PHH (Cooper and Chen, 1998; Newsted et al., 1995). The WHO fish TEF for PCB126 underestimated by 140-fold the relative potency (ReP) of PCB126 to induce ethoxyresorufin-*O*-deethylase (EROD) activity at environmentally relevant doses in early life stages of *F. heteroclitus* exposed by IVi injection (Rigaud et al., 2013). Moreover, in the same study, the slope of the dose-response relationship for prey capture ability was steeper for PCB126 than for TCDD and therefore, the TEF approach appeared inappropriate for this neurobehavioral response.

In Lake Ontario, one major source of DLCs was the commercially produced mixture of PCBs named Aroclor 1254 (Hu et al., 2011). In addition to dioxin-like PCBs, this commercial mixture contains non-dioxin-like (NDL) PCBs that do not bind selectively to the AHR but could possibly interact with DLCs. Developmental exposure to individual NDL PCBs and to complex Aroclor mixtures induce neurotoxic effects and behavioral alterations in mammals (Kodavanti and Ward, 2005; Llansola et al., 2010; Piedrafita et al., 2008). Various mechanisms of neurotoxic action have been proposed for these compounds including alterations in intracellular signaling pathways, neurotransmitters, thyroid hormones and oxidative stress

(Kodavanti et al., 2011). Long-term exposure of adult zebrafish (*Danio rerio*) to a diet spiked with an environmentally relevant mixture of four NDL PCBs induced behavioral disruptions in both adults and their offsprings (Péan et al., 2013). To the best of our knowledge, however, the neurotoxicity of single NDL PCBs and Aroclor mixtures has not been assessed in fish embryos.

In the present paper, the sublethal embryotoxicity and the ReP to TCDD of two additional DLCs were tested in *F. heteroclitus* exposed by IVi injection. The 3,3',4,4'-tetrachlorobiphenyl (PCB77) and the 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PnCDF) were selected because of their historic contribution to the decline of the Lake Ontario lake trout (Cook et al., 2003). According to the WHO TEFs (Van den Berg et al., 1998), PCB77 is the second most toxic dioxin-like PCB in fish after PCB126 and 2,3,4,7,8-PnCDF is the most toxic PCDF in fish. The sublethal embryotoxicity of two individual NDL PCBs was also assessed: the 2,2',5,5'-tetrachlorobiphenyl (PCB52) and the 2,3,3',4',6-pentachlorobiphenyl (PCB110) were selected because of their presence in Aroclor 1254 (Schulz et al., 1989), their relatively high neurotoxic equivalent value in mammals (respectively 0.699 and 0.971) (Simon et al., 2007) and their abundance in tissues of Lake Ontario lake trout in the late 70s (Huestis et al., 1996). Our objective was to assess whether environmentally realistic doses of these individual NDL PCBs were able to induce sublethal and/or neurobehavioral effects in *F. heteroclitus* early life stages. Finally, the sublethal embryotoxicity of Aroclor 1254 was evaluated. The observed toxicity of this PCB mixture was compared to that predicted from concentrations of PCB126, using the ReP estimated for *F. heteroclitus* for this potent dioxin-like PCB (Rigaud et al., 2013).

2.4 Materials and methods

2.4.1 Selection of doses, preparation and characterization of exposure solutions

The WHO reference TEF in fish for 2,3,4,7,8-PnCDF is 0.5 (Van den Berg et al., 1998), but RePs greater than 1 were already reported in fish for this compound, using EROD induction as the reference endpoint (Parrott et al., 1995; Parrott, 1997). In the present work, exposure doses (5, 20, 80, 320 and 1280 pg g^{-1} ww) similar to TCDD doses causing sublethal effects in *F. heteroclitus* (Rigaud et al., 2013) were chosen for 2,3,4,7,8-PnCDF. The lowest doses of 2,3,4,7,8-PnCDF are in the same range as measured concentrations for this compound in lake trout females or eggs from Lake Ontario during the late 1980s (Cook et al., 2003), i.e. 16.5 and 4.8 pg g^{-1} ww, respectively. PCB77 causes mortality in rainbow trout early life stages but is 25-50-fold less potent than PCB126 (Walker and Peterson, 1991). Selected doses of PCB77 (7.8, 15.6, 31.2, 62.5 and 125 ng g^{-1} ww) are 25-fold higher than doses of PCB126 causing sublethal effects in *F. heteroclitus* (Rigaud et al., 2013). The lowest exposure dose of PCB77 is in the same order of magnitude as measured concentrations in lake trout females or eggs from Lake Ontario in 1988, i.e. 3.8 and 1.3 ng g^{-1} ww, respectively. For PCB52 and PCB110, exposure doses (50, 200, 600, 1800 and 5400 ng g^{-1} ww) were based on data from Huestis et al. (1996) who reported concentrations around 300 and 500 ng g^{-1} ww for PCB52 and PCB110, respectively, in 4-year old lake trout collected in 1977 in Lake Ontario.

Aroclor 1254 (technical mixture, lot 5428), 2,3,4,7,8-PnCDF (in toluene, 1 mL, 5 µg mL^{-1}, 100% purity), PCB77 (solid form, 100% purity), PCB52 (solid form, 100% purity) and PCB110 (solid form, 99.7% purity) were all purchased from AccuStandard (New Haven, CT, USA). The triolein was obtained from Sigma-Aldrich (St-Louis, MO, USA). Triolein is an unsaturated triglyceride previously used as a vehicle solvent in fish embryotoxicity studies involving, for example, lake trout, rainbow trout or the mummichog (Carvalho and Tillitt, 2004; Rigaud et al., 2013; Wilson and Tillitt, 1996).

Stock solutions of PCB77, PCB52 and PCB110 were prepared by dissolving a known mass in triolein at nominal concentrations of 1.25 μg μL^{-1}, 13.5 μg μL^{-1} and 13.5 μg μL^{-1}, respectively. For 2,3,4,7,8-PnCDF, toluene was eliminated by evaporation and replaced by 500 μL of triolein to reach a nominal concentration of 10 μg mL^{-1}. A solution of approximately 100 mg mL^{-1} (sum of PCBs, nominal) of Aroclor 1254 in triolein was also prepared. Concentrations were validated by high (2,3,4,7,8-PnCDF and PCB77) or low (PCB52 and PCB110) resolution gas chromatography-mass spectrometry (GC-MS) in Laboratories of Expertise in Aquatic Chemical Analyses (LEACA, Fisheries and Oceans Canada, Maurice Lamontagne Institute, QC, and Institute of Ocean Sciences, BC, Canada). The purity of triolein was verified using high resolution GC-MS (Rigaud et al., 2013). Aroclor 1254 was characterized for PCB congeners using a gas chromatograph model HP5890 II (Agilent Technologies, Mississauga, ON, Canada) interfaced to an Autospec Ultima double-focusing magnetic sector mass spectrometer (Micromass, Manchester, United Kingdom). PCB analysis was performed with 55 to 60 m DB-5 and CP-19 columns to achieve optimal separation of PCB congeners.

2.4.2 Fish and egg care

F. heteroclitus adults (200 females and 100 males) were captured in May 2009 and 2010 in Horton's Creek (Miramichi, NB, Canada). Two groups of 150 fish were maintained under controlled photoperiod (16 h:8 h) in 420 L fiberglass tanks with flowing 25‰ salt water (St. Lawrence Estuary water filtered through silicate sand with a grain size of 0.8-1.2 mm). Fish were fed with pelleted cichlid food (Nutrafin®, Montréal, QC, Canada), dried shrimps and *Tubifex* worms. Water temperature was raised progressively from approximately 10 to 19°C to stimulate gonad maturation (Boyd and Simmonds, 1974). Artificial spawning substrates were placed in tanks at the end of the day and eggs were collected and randomly pooled into Petri dishes the next morning. Only normal and fertilized eggs at developmental stages 9-11 (Armstrong and Child, 1965) were retained for embryotoxicity tests.

2.4.3 Embryos exposure, incubation and hatching

Embryos exposures were performed on day 0 post-fertilization (DPF). Serial dilutions of Aroclor 1254, 2,3,4,7,8-PnCDF, PCB77, PCB52 and PCB110 in triolein were prepared from stock solutions (see section 2.4.1). Normal and fertilized eggs were divided randomly into seven groups of 35 embryos. For each compound, the experimental design included two control groups (non-injected and injected with triolein) and five groups injected with increasing doses of chemicals. Embryos were exposed by nano-injection of 2 nL of the desired triolein solution, as previously described in Rigaud et al. (2013). For Aroclor 1254 and PCB77, the injected volume was set to 4 nL instead of 2 nL to deliver enough of these compounds into the embryos. Preliminary experiments involving embryos injected with 2, 4 or 8 nL of pure triolein were performed to confirm that injected volumes up to 8 nL had no effects on survival and normal development of control embryos and larvae (data not shown). Moreover, embryos injected with a unique dose (1250 pg g^{-1} ww) of PCB126 but with different volumes (2, 4 or 8 nL) displayed no differences in terms of embryotoxic response (mortality, stunted growth or EROD activity, data not shown). For experiments involving DLCs (i.e. 2,3,4,7,8-PnCDF and PCB77) and Aroclor 1254, a positive control group was also included: positive controls consisted of embryos exposed by nano-injection to a unique dose of TCDD (160 pg g^{-1} ww, injected volume equal to 4 nL). A stock solution of TCDD in triolein was prepared and its concentration validated as described in Rigaud et al. (2013).

After treatment, embryos were incubated for 13 days at 22.5°C, under natural photoperiod (14 h:10 h), in 24-well Costar® polystyrene microplates (Corning Inc. Life Sciences, Lowell, MA, USA). The order of treated embryos within a plate was randomized, and the embryos were disposed individually on moist filter paper and monitored daily for mortality. Hatching was triggered after 13 DPF by adding 25‰ salt water into the wells. After 1 h, all unhatched and hatched embryos were transferred individually into 20 mL glass vials filled with 15 mL of 25‰ salt water until 16 DPF. Hatching success and mortality were monitored daily between 13 and 16 DPF, as water was renewed. Embryos were not fed during incuba-

tion. Salt water used for embryo incubation was from the same source as that used for adults (see section 2.4.2) but was additionally filtered (0.1 µm pore size) and UV-sterilized.

2.4.4 Locomotor activity

For each compound, the basal locomotor activity of 56 larvae (N = 8 per treatment, excluding the positive control group) was recorded during three successive periods of 5 min using a Zebrabox® (ViewPoint Life Sciences Inc., Montréal, QC, Canada), as previously described in Rigaud et al. (2013). The first and third period were recorded in the dark, under infrared light. The second 5 min period was recorded under white light to monitor larval reaction to a visual stimulus (MacPhail et al., 2009). Data obtained were expressed as three variables that represent basal locomotor activity of each larva: the rate of travel (i.e. the average swimming speed over each 5 min period, mm s^{-1}), the active swimming speed (i.e. the average swimming speed over each 5 min period, but during active time only, mm s^{-1}), and the inactivity (percent of the time the larva spent immobile). When the second 5 min period under white light began, mummichog larvae typically stopped moving for a few seconds and then alternated short movements and resting periods. The number and duration of the resting periods (considering only the periods of immobility lasting at least 2 s) were counted. The first resting period which occurred just after the light was turned on was defined as the recovery latency period (Rigaud et al., 2013).

2.4.5 Predatory capacities

Just after the locomotor activity assay, one larva from each treatment (excluding the non-injected control group and the positive control group) was randomly disposed in 6 adjacent wells of eight 48-well Costar® polystyrene microplates (N = 8 larvae per treatment). Microplates were prepared before the experiments by adding 20 *Artemia fransciscana* nauplii in each of the 6 adjacent wells (Gahtan et al., 2005). For each microplate, larval preda-

tion on nauplii was recorded during 5 min using a digital camera (Canon VIXIA HV30 HD Camcorder®, Canon Canada Inc., ON, Canada) under fluorescent light. The digital records were used to count *a posteriori* the number of *Artemia* captured and the number of feeding strikes each 30 s during the 5 min assay. The linear relationships between number of *Artemia* in the wells and time (min, log-transformed) were assessed by least-square regression for each larva. The rate of decline of counted *Artemia* was used as an index of prey capture ability. The number of *Artemia* captured per feeding strike was used as an index of prey capture efficiency (Couillard et al., 2011; Rigaud et al., 2013).

2.4.6 Morphometry

All larvae were observed individually and randomly (without knowing the treatment of each of them) under a stereo microscope coupled to an ocular micrometer. Body length was measured. Malformations were recorded, with particular attention to craniofacial deformities reported in the embryos of various fish species (including *F. heteroclitus*) exposed to DLCs (Elonen et al., 1998; Hill et al., 2004a; Rigaud et al., 2013). Cranial morphology of larvae was scored depending on the severity of the deformity: 0 for a normal larva, 1 for a larva showing an intermediate craniofacial deformity and 2 for a severe craniofacial deformity. Craniofacial deformities were characterized by a shortened snout, jaw or forehead. The severe craniofacial deformity was characterized by an extremely shortened snout, almost hidden behind the ocular globes when looking at the larva in lateral view. In the intermediate craniofacial deformity, the snout was shortened and flattened compared to normal but was still visible in lateral view. Other deformities typical of the blue sac disease (BSD) syndrome (for example edemas or hemorrhages) (Spitsbergen et al., 1991) were also monitored.

2.4.7 EROD activity measurement

Fifteen to twenty unfed larvae per treatment (3 or 4 pools of 5 larvae) were frozen in liquid nitrogen at the end of the measurements and kept at -80°C until EROD activity analyses. EROD activity was measured by spectrofluorimetry in homogenates of 5 larvae as previously detailed in Rigaud et al. (2013), using an adaptation of the method originally described by Fragoso et al. (1998). Data were expressed as pmoles resorufin min^{-1} mg^{-1} protein.

2.4.8 Data processing and statistical analyses

To characterize the lot of Aroclor 1254 used in this study, the relative contribution (%) of each congener was determined in the triolein-Aroclor 1254 stock solution and compared to the previously published congener profiles in early production and late production Aroclor 1254 (Frame, 1999). A total of 28 congeners accounting for approximately 80% of the total PCBs and representing each approximately ≥0.5% of the total PCBs were selected for this comparison. The 28 congeners selected for the comparison with Frame's data are listed in the x-axis of fig. 12.

SAS® 9.2 (SAS Institute Inc., Cary, NC, USA) was used to perform statistical analyses. The significance level was set at $\alpha = 0.05$ for all tests. The Shapiro-Wilk test was used to assess the normality of the data before and after log-transformation. Log-transformed EROD activity was distributed normally and compared among treatments using a one-way ANOVA followed by a Tukey's multiple comparisons test. Other data (body length, locomotor activity, prey capture ability and efficiency) were not normally distributed and were compared among treatments using a non-parametric Kruskal-Wallis test (KW) followed, when significant, by a multiple comparisons Tukey's studentized range test on the ranked values (Conover and Iman, 1981). For data expressed as proportions of individuals (mortality, prevalence of craniofacial deformities and hatching success) comparisons were done with the Fisher's exact test (FE).

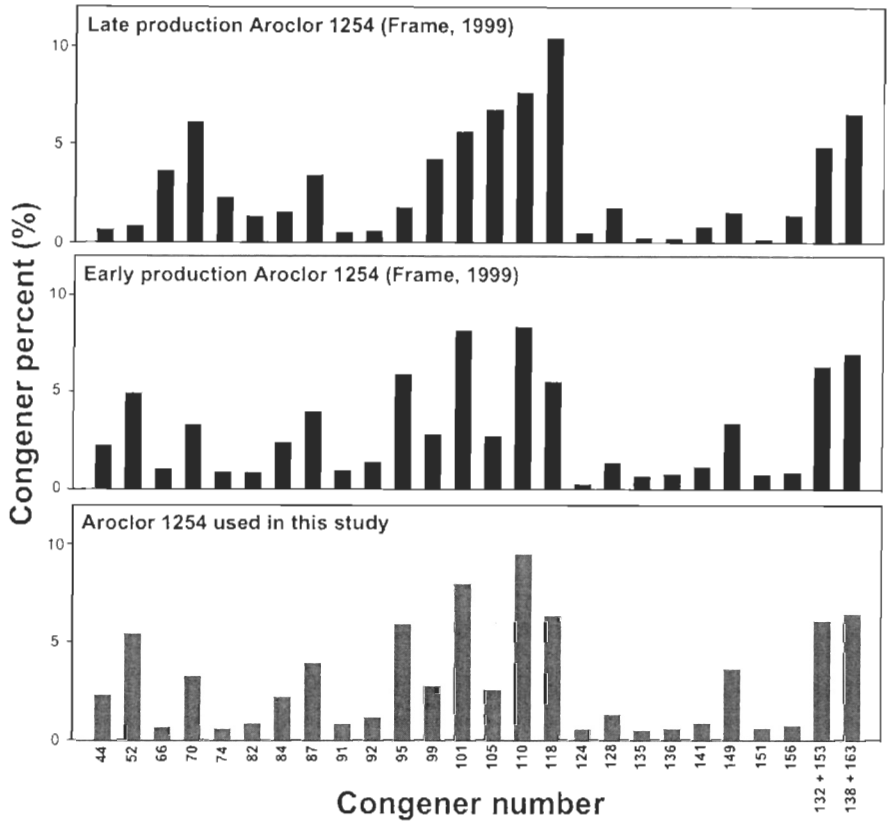

Figure 12: Comparison of the PCB congeners profile of the lot of Aroclor 1254 used in this study with data from Frame (1999).

Dose-response relationships for EROD activity and prey capture ability were examined by least square regression, with prior verification of the homoscedasticity and the normal distribution (Shapiro-Wilk statistic) of residuals. Data were expressed as percentages relative to triolein controls and log-transformed before regression analysis. For significant dose-relationships, homogeneity of slopes was tested using the general linear models procedure of SAS®. After confirmation of the homogeneity of slopes, the intercepts of the dose-response relationships of 2,3,4,7,8-PnCDF for EROD activity were compared to that of TCDD calculated in Rigaud et al. (2013) using an analysis of covariance (ANCOVA). The relative potency

of 2,3,4,7,8-PnCDF to TCDD was calculated by comparing the dose of each compound inducing an effect equivalent to the upper 95% confidence limit of control larvae for EROD induction (Parrott et al., 1995). These doses were calculated using the ANCOVA-adjusted regression lines for each compound. Relative potency for 2,3,4,7,8-PnCDF was defined as $ReP_{2,3,4,7,8\text{-PnCDF}} = \frac{Dose_{TCDD}}{Dose_{2,3,4,7,8\text{-PnCDF}}}$.

Bioassay-derived TCDD-TEQs were calculated for each Aroclor 1254 dose using observed EROD induction for each dose and regression parameters of TCDD for EROD activity calculated in Rigaud et al. (2013). They were compared to chemically derived TCDD-TEQs calculated from the concentration of PCB126 in Aroclor 1254 for each dose times it relative potency (0.71) to TCDD for EROD activity in *F. heteroclitus* larva (Rigaud et al., 2013). PCB77 was not included in chemically derived TCDD-TEQs calculations because a relative potency could not be estimated for this compound (see section 2.5.5).

2.5 Results

2.5.1 Chemical analyses and Aroclor 1254 characterization

Measured concentrations of 2,3,4,7,8-PnCDF, PCB77 and PCB52 in the triolein solutions (10.29 μg mL^{-1}, 1.59 μg μL^{-1} and 12.75 μg μL^{-1}) were close to nominal concentrations (10 μg mL^{-1}, 1.25 μg μL^{-1} and 13.5 μg μL^{-1}, respectively). For PCB110, however, the measured concentration was three times higher (36.6 μg μL^{-1}) compared to the nominal concentration (13.5 μg μL^{-1}).

The sum of the concentrations of all PCB congeners (N = 162) measured in the stock solution of Aroclor 1254 was 91 mg mL^{-1}. The recovery of PCB surrogates ranged from 78% to 123% with a mean value equal to 105 ± 11%. The congener profile of our Aroclor 1254 lot was close to that of early production Aroclor 1254 (Frame, 1999) (fig. 12). Measured

concentrations of PCBs in the stock solution and the highest exposure dose of Aroclor 1254 are presented in table 9.

2.5.2 Mortality and hatching success

Mortality rate did not differ between injected controls and non-injected controls, but was more variable in injected controls: 23% (3-30) and 11% (10-14), respectively (median value, Q1-Q3, for 5 experiments). 2,3,4,7,8-PnCDF, PCB77, PCB52, PCB110, Aroclor 1254 or TCDD (in positive control groups) did not cause any significant increase in mortality (data not shown).

Hatching success was not affected by triolein injection (data not shown). Hatching successes at 16 DPF for injected and non-injected controls were 97% (90-100) and 97% (97-100), respectively (median value, Q1-Q3, for 5 experiments). Hatching rate was significantly lower compared to controls (FE, $p \leq 0.05$, data not shown) at 13 DPF with the highest dose of 2,3,4,7,8-PnCDF (1280 pg g^{-1} ww), but not between 14 and 16 DPF. Hatching success was significantly reduced (FE, $p \leq 0.05$, data not shown) by the highest dose of PCB77 (125 ng g^{-1} ww) between 13 and 16 DPF. Hatching rate was not affected by Aroclor 1254, PCB52, PCB110 or TCDD (in positive control groups).

2.5.3 Body length and malformations

No significant differences in body length were observed between non-injected and triolein injected control groups in all experiments (data not shown). 2,3,4,7,8-PnCDF and Aroclor 1254 caused a significant (KW, $p \leq 0.05$) reduction in body length at ≥320 pg g^{-1} ww and 72 μg g^{-1} ww, respectively (fig. 13). Exposure to PCB77 significantly (KW, $p \leq 0.05$) reduced body length at 31.2 and 125 ng g^{-1} ww, but not at 62.5 ng g^{-1} ww (fig. 13). Body length was not affected by PCB52, PCB110 or TCDD (in positive control groups).

Table 9: Concentrations of selected PCBs congeners, sum of PCBs in the Aroclor 1254 stock solution and corresponding doses in the highest exposure dose of Aroclor 1254 for *F. heteroclitus* embryos.

	Aroclor 1254 stock solution (mg mL^{-1})	Highest exposure dose of Aroclor 1254 for *F. heteroclitus* embryos
ΣPCBs (162)[a]	91	72 μg g^{-1} ww
ΣPCBs (28)[b]	72	
Selected non-dioxin-like PCBs		
PCB52	5.0	4000 ng g^{-1} ww
PCB110	8.7	7000 ng g^{-1} ww
Selected dioxin-like PCBs		
PCB77	0.0077	6.2 ng g^{-1} ww
PCB126	0.0026	2056 pg g^{-1} ww

[a] Sum of all measured PCBs, N = 162 congeners.

[b] Sum of PCBs selected for the comparison with data from Frame (1999), N = 28 congeners.

Craniofacial malformations were not observed in control larvae or in larvae exposed to PCB52 or PCB110. The prevalence of severe craniofacial malformations was significantly increased only with the highest doses of 2,3,4,7,8-PnCDF (1280 pg g^{-1} ww) and PCB77 (125 ng g^{-1} ww), with respectively 15% (1-29) and 31% (15-47) of larvae affected (% affected and 95% confidence interval, FE, $p \leq 0.05$). Severe craniofacial malformations were not observed with Aroclor 1254. When intermediate craniofacial malformations were also considered, the prevalence of all craniofacial malformations was significantly increased by 2,3,4,7,8-PnCDF, PCB77 and Aroclor 1254 at doses of ≥320 pg g^{-1} ww, ≥31.2 ng g^{-1} ww and ≥36 μg g^{-1} ww, respectively (FE, $p \leq 0.05$, data not shown). Prevalence of edemas was only significantly increased by the highest dose of PCB77 (125 ng g^{-1} ww), with 16% (0-28) of larvae impacted (% affected and 95% confidence interval, FE, $p \leq 0.05$).

Prevalence of craniofacial deformities did not differ significantly among experiments

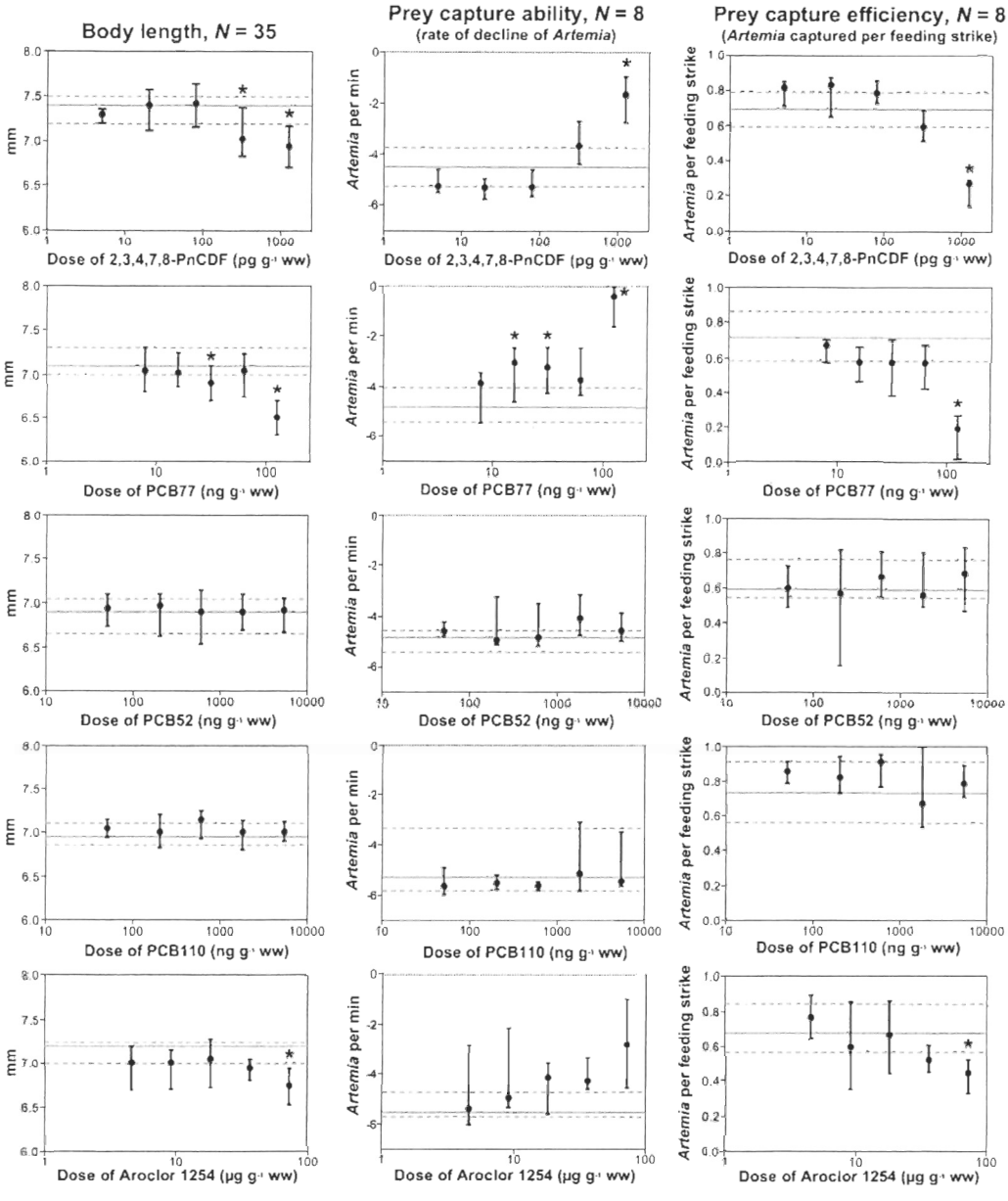

Figure 13: Effect of 2,3,4,7,8-PnCDF, PCB77, PCB52, PCB110 and Aroclor 1254 on body length, prey capture ability and prey capture efficiency of *F. heteroclitus* larvae at 16 DPF. Prey capture ability is expressed as the rate of decline of *Artemia* in the well, i.e. the slope of the relationship between the number of *Artemia* remaining in the well and time (logarithmically transformed). Prey capture efficiency is expressed as the number of *Artemia* captured per feeding strike. Values are presented as median (dots) and interquartile range (vertical bars, Q1-Q3). Solid and dotted horizontal lines represent median value and interquartile range for the triolein control group. The non-injected control group is not shown. Doses of Aroclor 1254 are expressed as ΣPCBs (N = 162 congeners) (see table 9). * Indicates a significant difference compared to the triolein control group (KW, $p \leq 0.05$).

in the TCDD-injected control groups (FE, $p > 0.05$). The prevalence of intermediate craniofacial malformations were 30% (15-46), 40% (19-61) and 9% (0-26) (% affected and 95% confidence interval) in experiments involving PCB77, Aroclor 1254 and 2,3,4,7,8-PnCDF, respectively.

2.5.4 Locomotor activity and predatory capacity

None of the studied compounds had an effect on locomotor activity (rate of travel, active swimming speed or inactivity) during light or dark periods (data not shown). No significant effect was detected on the recovery latency, or the number and the duration of the resting times during the light period (data not shown).

Prey capture ability was significantly (KW, $p \leq 0.05$) reduced by exposure to 2,3,4,7,8-PnCDF at the highest dose tested (1280 pg g^{-1} ww) and by PCB77 at ≥15.6 ng g^{-1} ww (except for the 62.5 ng g^{-1} ww dose) (fig. 13). Exposure to the highest doses of Aroclor 1254 tended to reduce prey capture ability, but it was not statistically significant, with high inter-individual variability (fig. 13). Prey capture ability was not altered by PCB52 or PCB110 (fig. 13). The highest doses of 2,3,4,7,8-PnCDF (1280 pg g^{-1} ww), PCB77 (125 ng g^{-1} ww) and Aroclor 1254 (72 µg g^{-1} ww) significantly (KW, $p \leq 0.05$) reduced prey capture efficiency (fig. 13). PCB52 and PCB110 did not affect prey capture efficiency. None of the studied compounds affected the number of feeding strikes attempted by the larvae over the 5 min predation assay (KW, $p > 0.05$, data not shown). The relationships between the number of prey captured and the number of feeding strikes showed that some larvae exposed to the highest doses of 2,3,4,7,8-PnCDF, PCB77 or Aroclor 1254 captured a relatively low number of *Artemia* despite attempting a large number of feeding strikes (fig. 14).

2,3,4,7,8-PnCDF reduced prey capture ability in a dose-dependent manner ($R^2 = 0.56$, $p \leq 0.05$, $F = 48.08$, DF = 38). Comparison of the dose-response relationships of 2,3,4,7,8-PnCDF to that calculated for TCDD in Rigaud et al. (2013) revealed that the slopes of the

dose-response relationships for prey capture ability were not homogenous ($p \leq 0.05$), and that the slope was steeper for 2,3,4,7,8-PnCDF (0.41 ± 0.06) than for TCDD (0.19 ± 0.07). The assumptions required for performing linear regression analysis (i.e. homoscedasticity, normality of residuals, etc.) were not respected for the relationships between prey capture ability and doses for PCB77 and Aroclor 1254.

2.5.5 EROD activity and estimation of relative potencies

As expected, EROD activity was not induced by the injection of triolein alone (data not shown). EROD activities of TCDD positive control groups were 3.50 ± 1.16, 3.28 ± 0.35 and 1.95 ± 0.65 pmoles resorufin min^{-1} mg^{-1} protein (mean ± standard deviation) in experiments involving 2,3,4,7,8-PnCDF, PCB77 and Aroclor 1254, respectively, and did not differ significantly among experiments (ANOVA, $p > 0.05$).

EROD activity increased with dose for both 2,3,4,7,8-PnCDF ($R^2 = 0.78$, $p \leq 0.05$, $F = 31.98$, DF = 9) and PCB77 ($R^2 = 0.42$, $p \leq 0.05$, $F = 12.17$, DF = 17) (table 10) with significant induction (ANOVA, $p \leq 0.05$) at ≥20 pg g^{-1} ww and ≥15.6 ng g^{-1} ww, respectively (fig. 15). Comparisons of the dose-response relationships of 2,3,4,7,8-PnCDF to that calculated for TCDD in Rigaud et al. (2013) revealed that the slopes were homogenous ($p > 0.05$) and that the intercepts were significantly different (ANCOVA, $p \leq 0.05$) (table 10). The ReP of 2,3,4,7,8-PnCDF to TCDD for EROD induction was estimated as 2.40. EROD activity increased with dose for Aroclor 1254 ($R^2 = 0.76$, $p \leq 0.05$, $F = 55$, DF = 17) (table 10) with significant (ANOVA, $p \leq 0.05$) induction at ≥18 µg g^{-1} ww (fig. 15). PCB52 and PCB110 did not cause EROD induction (fig. 15). The slopes of the dose-response relationships for PCB77 and Aroclor 1254 were significantly lower than the slope for TCDD ($p \leq 0.05$) (table 10). Thus, the relative potency of PCB77 and Aroclor 1254 could not be estimated.

For Aroclor 1254, the bioassay-derived TCDD-TEQs were lower than the chemically

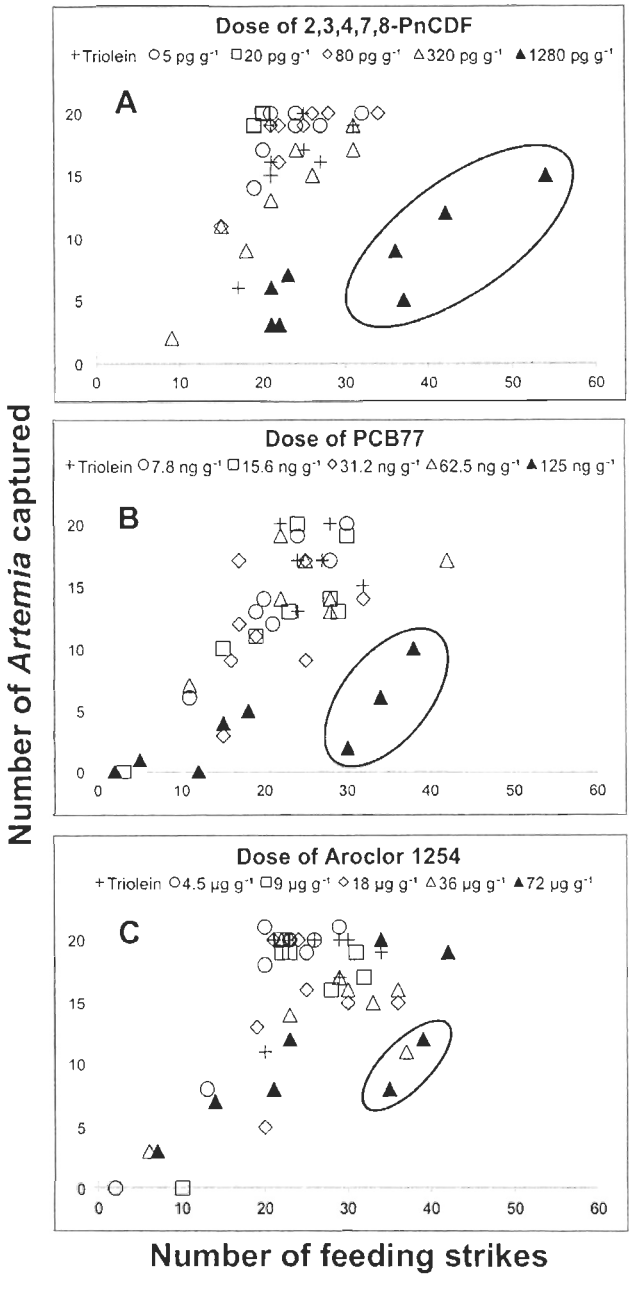

Figure 14: Relationships between the number of *Artemia* captured and the number of feeding strikes for each larva, following treatment with 2,3,4,7,8-PnCDF (A), PCB77 (B) or Aroclor 1254 (C). Ellipses circle some larvae capturing a relatively small number of prey despite attempting a large number of feeding strikes. $N = 8$ per dose.

Table 10: Regression parameters relating the log of the EROD activity (expressed as percentages relative to injected controls) and the log of the exposure dose for selected chemicals. Parameters of the comparison of the linear regressions to the one of TCDD by ANCOVA are also presented.

	Regression parameters[a]				ANCOVA versus TCDD[b]		ANCOVA-adjusted regression parameters[c]		
Chemicals	*a*	*b*	R^2	*p*	Slope	Intercept	*a*	*b*	R^2
TCDD[d]	0.62 ± 0.06	1.25 ± 0.13	0.92	<0.001			0.54 ± 0.05	1.41 ± 0.07	0.86
2,3,4,7,8-PnCDF	0.47 ± 0.08	1.77 ± 0.18	0.78	<0.001	>0.05	<0.01	0.54 ± 0.05	1.62 ± 0.12	0.86
PCB77	0.23 ± 0.07	1.39 ± 0.30	0.42	<0.01	<0.001				
Aroclor 1254	0.37 ± 0.05	1.84 ± 0.07	0.76	<0.001	<0.01				

[a] Model formula is: Log[y] = *a**Log[x] + *b*, where *a* is the slope, *b* the intercept, x the dose (log-transformed) and y is EROD activity (expressed as percentages relative to injected controls and log-transformed).

[b] Comparison of the parameters (slope and intercept) of the dose-response relationship with the one of TCDD, by ANCOVA. Intercepts were not compared when slopes were significantly differents ($p \leq 0.05$).

[c] ANCOVA-adjusted regression parameters (i.e. pooled slope and respective adjusted intercepts) are presented here for the only dose-response relationships with homogenous slopes, i.e. for 2,3,4,7,8-PnCDF versus TCDD.

[d] Regression parameters for TCDD were obtained from Rigaud et al. (2013).

derived TCDD-TEQs based on measured concentrations of PCB126 (tables 9 and 11). The discrepancy between the bioassay- and the chemically derived TCDD-TEQs increased from 4 to 12-fold with increasing doses of Aroclor 1254 (table 11).

2.6 Discussion

2.6.1 Embryotoxicity and relative potencies of dioxin-like compounds

Both 2,3,4,7,8-PnCDF and PCB77 caused embryotoxic responses in *F. heteroclitus* larvae similar to those observed with TCDD and PCB126 in Rigaud et al. (2013): in addition to classical AHR-related responses (EROD induction, craniofacial deformities and reduction in body length), all these compounds altered predatory capacities without reducing basal locomotor activity. As observed with TCDD and PCB126, some larvae exposed to the highest doses of 2,3,4,7,8-PnCDF (1280 pg g^{-1} ww) or PCB77 (125 ng g^{-1} ww) captured relatively few prey while attempting a large number of feeding strikes (fig. 14), which is consistent with visual impairment previously proposed by Carvalho and Tillitt (2004). As observed with PCB126 (Rigaud et al., 2013), the WHO fish TEFs based on trout embryolethality were not adequate to assess the sublethal toxicity of 2,3,4,7,8-PnDF and PCB77 in early life stages of *F. heteroclitus*. Even if species-specific TEFs were generated for induction of EROD activity in *F. heteroclitus*, the TCDD-TEQ approach was invalid for neurobehavioral responses because dose-response curves for the compounds tested were not parallel to that of TCDD.

The ReP of 2,3,4,7,8-PnCDF (2.40) for EROD activity in *F. heteroclitus* was markedly higher compared to the WHO fish TEF (0.50) (Van den Berg et al., 1998). A wide range of RePs (0.12-8.10) has been reported for 2,3,4,7,8-PnCDF in several fish species for both *in vivo* and *in vitro* measured AHR-related biochemical endpoints and mortality (table 12). When considering *in vivo* data only, the ReP (2.0) of 2,3,4,7,8-PnCDF for EROD activity in adult rainbow trout (Parrott et al., 1995) was close to the value reported in the present study (table 12). In contrast, RePs for EROD and CYP1A protein induction in the common carp

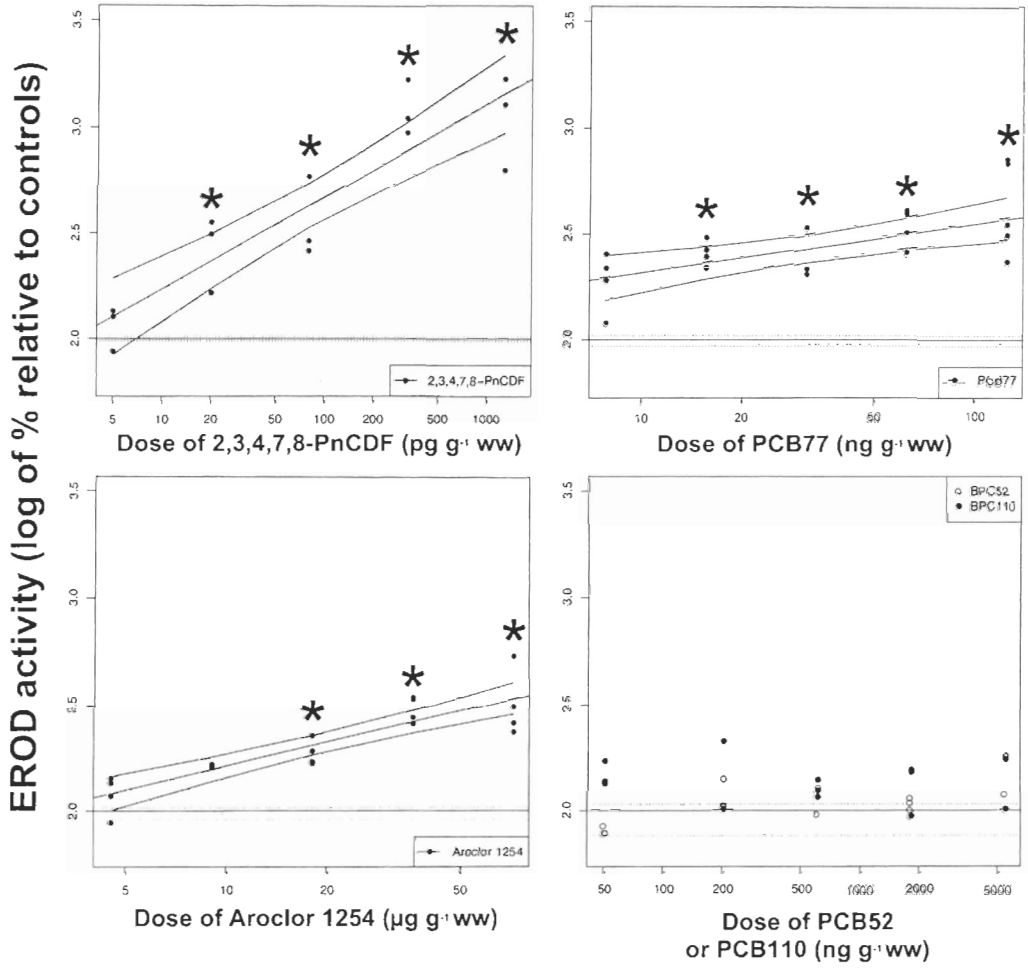

Figure 15: Effect of 2,3,4,7,8-PnCDF, PCB77, PCB52, PCB110 and Aroclor 1254 on EROD activity (log-transformed, N = 3-4) of *F. heteroclitus* larvae at 16 DPF. EROD activity was expressed as percentages relative to triolein controls and log-transformed prior to regression analysis. Solid and dotted horizontal lines represent median value and interquartile range for the triolein control group. The non-injected control group is not shown. Doses of Aroclor 1254 are expressed as ΣPCBs (N = 162 congeners) (see table 9). * Indicates a significant difference compared to the triolein control group (ANOVA, $p \leq 0.05$).

Table 11: Comparison of bioassay- and chemically derived TCDD-TEQs corresponding to each Aroclor 1254 exposure dose.

Exposure dose of Aroclor 1254[a] ($\mu g\ g^{-1}$ ww)	Bioassay-derived[b] TCDD-TEQs ($pg\ g^{-1}$ ww)	Chemically derived[c] TCDD-TEQs ($pg\ g^{-1}$ ww)	Fold difference[d]
4.5	22 ± 7	91	4.1
9	35 ± 1	182	5.2
18	45 ± 11	365	8.1
36	98 ± 23	730	7.5
72	121 ± 84	1460	12.1

[a] Sum of all measured PCBs, N = 162 congeners.

[b] Bioassay-derived doses in terms of TCDD-TEQs were calculated using observed EROD activity induction following exposure of *F. heteroclitus* embryos to Aroclor 1254 and regression parameters for TCDD (table 10).

[c] Chemically derived doses in terms of TCDD-TEQs were determined from the concentration of PB126 measured in the highest exposure dose of Aroclor 1254 (table 9) times its relative potency to TCDD for EROD activity in *F. heteroclitus* larva (0.71) calculated in Rigaud et al. (2013).

[d] Fold difference between bioassay- and chemically derived TCDD-TEQs.

(*Cyprinus carpio*) were lower (0.36 and 0.12, respectively) and closer to the WHO fish TEF (van der Weiden et al., 1994). Interspecies variability in fish in terms of toxicodynamics and toxicokinetics, of AHR isoforms (Garner et al., 2013), and of number, type and expression pattern of AHR pathway genes (Zhou et al., 2010), as well as endpoint selection and choice of target tissues can contribute to the observed differences in RePs. Interspecies variability also exists in mammals, as RePs ranging from 0.0065 to 3.7 were reported for 2,3,4,7,8-PnCDF for both *in vivo* and *in vitro* studies (Haws et al., 2006). High interspecies variability as well as high ReP values (> 10) were also reported for 2,3,4,7,8-PnCDF in birds in both *in vitro* and *in ovo* embryolethality studies (Cohen-Barnhouse et al., 2011; Hervé et al., 2010a,b,c).

In the present study, 2,3,4,7,8-PnCDF was at least as potent as TCDD in reducing prey capture ability of 16 DPF *F. heteroclitus* larvae, as similar effective doses were observed for these two compounds (i.e., 1280 pg g^{-1} ww) (Rigaud et al., 2013). However, no ReP was estimated for this endpoint because of different slopes (steeper dose-response for 2,3,4,7,8-PnCDF). A similar finding was previously reported by Rigaud et al. (2013) for PCB126 and it was associated with different transcriptional pathways: PCB126 appeared to induce antioxidant responses by inducing superoxide dismutase (*sod2*) expression, while TCDD did not. Different slopes of dose-response curves could be observed with two DLCs sharing a common mode of action (AHR activation) but displaying different pathways of effects including cross talk mechanisms that involve the AHR nuclear translocator (ARNT) (Olufsen and Arukwe, 2011), production of reactive oxygen species (ROS) (Arzuaga et al., 2006), as well as signaling pathways (Gjernes et al., 2012) that can alter organismal responses.

The ReP of PCB77 for EROD activity could not be estimated due to non-parallelism of dose-response relationships between TCDD and PCB77. A slight inhibition of EROD activity was observed for 3 of 4 of the larvae with the highest dose of PCB77 tested (125 ng g^{-1} ww, Figure 15). Inhibition of EROD and CYP1A protein induction was previously described in scup (*Stenotomus chrysops*) treated with high doses of PCB77 (Gooch et al., 1989; White et al., 1997). The loss of EROD activity at high doses of PCB77 was also observed *in vitro* in

Table 12: Overview of some relative potencies of 2,3,4,7,8-PnCDF versus TCDD determined from *in vivo* or *in vitro* studies and for AHR-related biochemical endpoints and mortality in fish.

Endpoint and species	Tissues	Route of exposure	Relative potency	Reference
EROD (*in vivo*)				
Fundulus heteroclitus	**Whole body (larvae)**	**IVi[a] injection (triolein)**	**2.40**	**This study**
Oncorhynchus mykiss	Liver (adults)	Oral	2.00[c]	Parrott et al. (1995)
Cyprinus carpio	Liver	IP[b] injection (peanut oil)	0.36[c]	van der Weiden et al. (1994)
EROD (*in vitro*)				
Oncorhynchus mykiss	Liver cells (RTL-W1)	Aqueous (DMSO)	1.90 ± 0.40	Clemons et al. (1994)
CYP1A protein (*in vivo*)				
Cyprinus carpio	Liver	IP[b] injection (peanut oil)	0.12[c]	van der Weiden et al. (1994)
CYP1A mRNA (*in vitro*)				
Oncorhynchus mykiss	Gonadal cells (RTG-2)	Aqueous (DMSO)	0.13	Zabel et al. (1996)
Danio rerio	Liver cells (ZF-L)	Aqueous (DMSO)	8.10 ± 2.00	Henry et al. (2001)
Mortality				
Oncorhynchus mykiss	Embryo/Larvae	Aqueous (Toluene)	0.35	Bol et al. (1989)
		IVi[a] injection (liposomes)	0.36 (0.25-0.91)	Walker and Peterson (1991)

[a] Intravitelline.

[b] Intraperitoneal.

[c] Based on internal dose measured in liver.

both fish and rat liver cells (Edwards et al., 2007; Hahn et al., 1993) and it has been attributed in fish to oxidative damage to the CYP1A protein (Schlezinger et al., 1999, 2000).

2.6.2 Embryotoxicity of non-dioxin-like PCB congeners, PCB52 and PCB110

Environmentally relevant doses of PCB52 and PCB110, two di-ortho-substituted PCBs (non-dioxin-like congeners), induced no significant behavioral alterations in *F. heteroclitus* larvae, including locomotor activity, reaction to a light stimulus, or predatory capacities. Because prey capture ability was altered by all DLCs tested and not by NDL PCBs, this response appears AHR-dependent.

PCB52 and PCB110 are known as potentially neurotoxic in mammals (Eriksson and Fredriksson, 1996; Kodavanti et al., 1996; Llansola et al., 2010; Pessah et al., 2006). However, to our knowledge, no studies have reported the ability of single ortho-substituted PCB to induce behavioral alterations in fish. Long-term exposure to a diet spiked with an environmentally relevant mixture of NDL PCBs (including PCB52) induced behavioral disruptions (nocturnal behavior alteration, hyperactivity in a challenging situation and changes in the vertical positioning) in both zebrafish adults and their offspring (Péan et al., 2013). Compared to intravitelline injection measuring the direct toxic effects of contaminants to the developing embryo, exposure of the mother to persistent organic pollutants such as PCBs could also affect transfer of nutrients or hormones to the egg which could negatively impact embryo development (Brooks et al., 1997).

2.6.3 Sublethal embryotoxicity of Aroclor 1254

Aroclor 1254 caused a pattern of sublethal embryotoxic responses similar to those previously observed with DLCs (TCDD, PCB126, PCB77 and 2,3,4,7,8-PnCDF) in this study or in Rigaud et al. (2013). The concentration of PCB77 (6.2 ng g^{-1} ww) in the highest exposure dose of Aroclor 1254 was lower than the lowest exposure dose of PCB77 alone (7.8

ng g^{-1} ww) that produced no significant effect on *F. heteroclitus* embryos. It suggests that the contribution of PCB77 to the observed embryotoxicity of Aroclor 1254 is almost equal to zero. Due to the combination of its concentration and its high TEF compared to other dioxin-like or mono-ortho-substituted PCBs, PCB126 is the most important contributor to total TCDD-TEQs in Aroclor mixtures (Bhavsar et al., 2008b; Zhang et al., 2012). In this study, the chemically derived TCDD-TEQs, based on PCB126 concentrations and using a species-specific ReP (Rigaud et al., 2013), overestimated the potency of Aroclor 1254 to induce EROD activity in *F. heteroclitus* larvae. At the highest exposure dose of Aroclor 1254, PCB52 and PCB110 were at concentrations of 4000 and 7000 ng g^{-1}, respectively (Table 9), close to the highest nominal doses used for single exposure of *F. heteroclitus* to these two congeners (i.e. 5400 ng g^{-1} ww) and causing no behavioral or morphological alterations. Thus, it is unlikely that these congeners induced the sublethal responses observed in *F. heteroclitus* treated with Aroclor 1254, which were similar to those observed in this study and previous studies in *F. heteroclitus* treated *in ovo* with DLCs (Rigaud et al., 2013).

Bioassay-derived TCDD-TEQs were lower than chemically derived TCDD-TEQs and this difference increased with increasing doses of Aroclor 1254. In a complex mixture, interactions among compounds can affect the shape of the dose-response relationship. As the concentration of agonists in the sample increases (dioxin-like PCBs in the case of Aroclor 1254), so does the concentration of antagonists, leading to a more gentle slope and a lower maximal response (Villeneuve et al., 2000). Partial or weak agonists can complicate accurate TCCD-TEQs determination because they occupy a large number of receptors without eliciting a detectable response: this can competitively block the effects of agonists (Whyte et al., 2004). Harris et al. (1994) found that a complex mixture of PCDDs, PCDFs and PCBs (both dioxin-like and non-dioxin-like) was approximately 10-times less embryotoxic for Japanese medaka embryos than the exact same mixture without its PCDDs, PCDFs and NDL PCBs. In addition to hypothesizing antagonistic interactions leading to competition for the AHR to explain this difference, these authors showed that the uptake of radiolabeled PCB126 from the rearing medium to medaka embryos was reduced in the presence of high concentrations of

the complex mixture. In the present study, Aroclor 1254 was directly injected into the yolk of *F. heteroclitus* embryos to avoid differential uptake of different congeners across the chorion. However it is still possible that the toxicity of Aroclor 1254 was limited pharmacokinetically: the presence of high concentrations of lipophilic congeners can alter compound partitioning and tissue distribution, leading to reduced concentrations of dioxin-like compounds in target tissues and lower EROD induction (Walker et al., 1996a).

This study highlights the limitations of using TEFs generated from limited toxicity data mostly in salmonid species for risk assessment in phylogenetically distant fish species. It supports the need for further studies on the variability of RePs of DLCs in a range of different fish species and for various sublethal toxicity endpoints with standardized protocols. Finally, the TCDD-TEQ approach should also be used with caution in fish exposed in the environment to complex mixtures of chemicals because interactions with other classes of chemicals could significantly alter the risk of deleterious impacts of DLCs. In the wild, fish are exposed to mixtures more complex than Aroclor 1254, which include a large variety of lipophilic and potentially neurotoxic compounds such as PCBs, PCDDs, PCDFs, organochlorine pesticides or brominated compounds (such as polybrominated diphenyl ethers). Experiments involving exposure of *F. heteroclitus* embryo and larvae to complex organic extracts obtained from highly contaminated whole fish are needed to assess if results similar to those obtained with Aroclor 1254 can be found with more environmentally realistic mixtures.

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ARTICLE III

TEMPORAL VARIATIONS IN EMBRYOTOXICITY OF LAKE ONTARIO AMERICAN EEL (*ANGUILLA ROSTRATA*) EXTRACTS TO DEVELOPING *FUNDULUS HETEROCLITUS*

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Contribution de chaque auteur - **C. Rigaud** : conception du projet, expérimentation et analyse des données, rédaction de l'article et conception des figures ; **C. M. Couillard** : conception du projet, supervision, aide à l'expérimentation et à la rédaction ; **J. Pellerin** : supervision et aide à la rédaction ; **B. Légaré** : aide à l'expérimentation ; **J. D. Byer** : analyses chimiques ; **M. Alaee** : analyses chimiques ; **M. Lebeuf** : analyses chimiques et aide à l'expérimentation ; **J. M. Casselman** : sélection et mesures biologiques sur les anguilles ; **P. V. Hodson** : direction et coordination du projet CRSNG, supervision et aide à la rédaction.

3.1 Résumé

Le recrutement des juvéniles de l'anguille américaine (*Anguilla rostrata*) vers le lac Ontario (LO), Canada, a décliné drastiquement depuis les années 1980. Afin d'explorer la contribution possible à ce déclin des polluants organiques persistants (POP) transférés par les femelles aux œufs, la présente étude mesure les variations temporelles de la toxicité de mélanges organiques complexes de POP extraits d'anguilles américaines capturées dans le LO en 1988, 1998 et 2008, chez les embryons de *Fundulus heteroclitus* exposés par injection intravitelline (IVi). Les extraits de 1988 et 1998 ont été les plus toxiques, provoquant un patron de réponses embryotoxiques sous-létales similaire à celui précédemment observé chez des embryons de *F. heteroclitus* exposés à des composés apparentés aux dioxines individuels : retard de croissance, malformations crânio-faciales, induction de l'activité EROD et réduction de la capacité de prédation. Le potentiel toxique des extraits a décliné avec le temps ; le seul effet significatif observé avec les extraits de 2008 était l'induction de l'activité EROD. Les EQT-TCDD dérivés des données chimiques, calculés à partir des concentrations de certains composés apparentés aux dioxines dans les extraits et de leurs PTR respectifs chez *F. heteroclitus*, ont surestimé la capacité des extraits à induire EROD, possiblement en raison d'interactions entre les différents POP. Les autres POP mesurés dans les extraits d'anguilles (BPC non-coplanaires, PBDE et pesticides organochlorés) n'ont pas semblé être d'importants contributeurs à l'embryotoxicité observée. La toxicité des mélanges organiques complexes de POP accumulés par les anguilles du LO a pu être sous-estimée en raison de plusieurs facteurs, incluant les pertes chimiques durant la préparation des extraits, ainsi que l'absence d'évaluation de leurs effets à long terme. Dans l'ensemble, nos résultats supportent l'hypothèse selon laquelle la contamination du LO par les composés apparentés aux dioxines aurait représenté une menace pour la population de l'anguille d'Amérique en altérant des paramètres pertinents d'un point de vue environnemental, telle que la capacité de prédation des larves. Ces résultats soulignent l'importance de tester l'embryotoxicité des composés apparentés aux dioxines chez les jeunes stades de vie des anguilles, d'explorer les effets à

long terme d'extraits organiques d'anguilles sur les jeunes stades de vie des poissons et de développer des biomarqueurs pour évaluer les altérations potentielles chez les jeunes anguilles échantillonnées sur le terrain.

3.2 Abstract

Recruitment of American eel (*Anguilla rostrata*) juveniles to Lake Ontario (LO), Canada, declined significantly since the 1980s. To investigate the possible contribution of maternally transferred persistent organic pollutants (POPs) to this decline, this study measured temporal variations in the toxicity of complex organic mixtures extracted from LO American eels captured in 1988, 1998 and 2008 to developing *Fundulus heteroclitus* exposed by intravitelline (IVi) injection. The 1988 and 1998 eel extracts were the most toxic, causing a pattern of sublethal embryotoxic responses similar to those previously reported in *F. heteroclitus* embryos exposed to single dioxin-like compounds (DLCs): stunted growth, craniofacial deformities, induction of EROD activity, and reduced predatory capacities. The potency of these extracts declined over time; the only significant effect of the 2008 eel extracts was EROD induction. The chemically derived TCDD-TEQs of eel extracts, calculated using measured concentrations of some DLCs and their relative potencies for *F. heteroclitus*, overestimated their potency to induce EROD activity possibly due to interactions among POPs. Other POPs measured in eel extracts (non-dioxin-like PCBs, PBDEs and organochlorinated pesticides) did not appear to be important agonistic contributors to the observed toxicity. The toxicity of the complex mixtures of POPs accumulated by LO eels may have been underestimated as a result of several factors, including the loss of POPs during extracts preparation and the non-assessment of their potential long-term effects. Overall, our results support the hypothesis that contamination of LO with DLCs may have represented a threat for the American eel population through ecologically relevant effects such as altered larval prey capture ability. These results prioritize the need to assess early life stage (ELS) toxicity of DLCs in *Anguilla* species, to investigate long-term or delayed effects of complex eel extracts to ELS

of fish and to further develop biomarkers to assess potential effects in eel ELS sampled in the field.

3.3 Introduction

Recruitment of juvenile American eels (*Anguilla rostrata*) to Lake Ontario (LO), Canada, declined significantly since the 1980s, and almost no recovery has ben observed to date (COSEPAC, 2012). Possible causes include habitat modifications, overfishing, oceanic changes and chemical contamination (Castonguay et al., 1994). Except for stocked eels, LO contains large and highly fecund female eels, presumably a dominant source of eggs for the entire species (Castonguay et al., 1994; Dutil et al., 1985). As a benthic top predator fish species that is long-lived and lipid-rich, American eel bioaccumulate large amounts of lipophilic persistent organic pollutants (POPs) such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and organochlorinated pesticides (OCPs) (Byer et al., 2013a,b). The maternal transfer of these POPs to eggs and their deleterious effects to early life stages (ELS) has been proposed as a possible contributor to the recruitment failure of Atlantic eel populations (Couillard, 2009; Palstra et al., 2006; Robinet and Feunteun, 2002).

Among POPs accumulated by eels, dioxin-like compounds (DLCs) include PCDDs (75 congeners), PCDFs (135 congeners) and non-ortho-substituted (also named coplanar) PCBs (4 congeners). In vertebrates, the toxicity of DLCs is modulated by the aryl hydrocarbon receptor (AHR) (Poland and Glover, 1976). Fish ELS exposed to high doses of DLCs are affected by the blue sac disease (BSD) syndrome characterized by cytochrome P4501A (CYP1A) induction, stunted growth, craniofacial deformities, edemas, hemorrhages and mortality (Spitsbergen et al., 1991). In LO, the collapse of lake trout (*Salvelinus namaycush*) during the mid 20th century was partially attributed to lethal embryotoxic concentrations of DLCs in eggs (Cook et al., 2003). Concentrations of DLCs in LO have declined since the

1980s (Gewurtz et al., 2009). However, sublethal effects of these compounds in fish ELS such as delayed growth or impaired prey capture ability may still impact lake trout populations (Carvalho et al., 2004; Carvalho and Tillitt, 2004). There is growing evidence that DLCs are able to significantly reduce prey capture ability of fish larvae at sublethal doses below those inducing the BSD syndrome, as recently shown in mummichog (*Fundulus heteroclitus*) and zebrafish (*Danio rerio*) (Chollett et al., 2014; Couillard et al., 2011; Rigaud et al., 2013, 2014). Behavioral responses such as locomotor activity or prey capture ability are generally sensitive to low levels of a variety of environmental contaminants, and can have major ecological significance by altering fitness, growth, migratory competence and recruitment success at the population level (Weis and Candelmo, 2012).

American eels collected in LO in 2008 had relatively high concentrations of POPs, namely PCDDs, PCDFs, PCBs, OCPs and PBDEs, compared to eels captured from other locations in Canada, but lower than historic values in migrating eels captured in the St. Lawrence estuary (Byer et al., 2013a,b; Castonguay et al., 1989; Hodson et al., 1994). Prior to 2000, concentrations of DLCs exceeded chronic toxicity thresholds historically documented for lake trout in LO, suggesting a possible effect on the quality of spawners and their eggs (Byer, 2013). Although concentrations of legacy POPs are decreasing as a consequence of environmental regulations, the concentrations of unmeasured emerging contaminants could possibly have increased in LO American eels in recent years, as documented in lake trout (Clement et al., 2012). In addition to chemical analyses, bioassays are needed to assess the hazard of deleterious impacts on developing ELS associated with the accumulation of a complex mixture of POPs in LO eel's fat. Considering the difficulties associated with producing ELS of American eels in the laboratory (Oliveira and Hable, 2010), this study explores the embryotoxicity of complex organic mixtures extracted from LO American eels captured between 1988 and 2008 to developing *Fundulus heteroclitus*, an euryhaline fish species which we have used as a surrogate for eel. Earlier studies showed that intravitelline (IVi) injections of sublethal doses of several DLCs historically found in LO (Cook et al., 2003) and of a complex mixture of PCBs (Aroclor 1254) cause induction of EROD activity, craniofacial

deformities, reduced body length and reduced prey capture ability in *Fundulus heteroclitus* larvae (Couillard et al., 2011; Rigaud et al., 2013, 2014).

The specific objectives of the present study were to assess whether (1) American eels accumulated enough POPs during the 80s and the 90s to cause embryotoxicity to developing *F. heteroclitus*, (2) the toxicity of eel extracts varied among years, and (3) toxicity was predictable on the basis of the measured concentrations of DLCs in these extracts. Spontaneous locomotor activity and prey capture ability of mummichog larvae were measured in parallel to more traditional AHR-related endpoints: mortality, hatching success, malformations, growth and CYP1A induction, as indicated by ethoxyresorufin-*O*-deethylase (EROD) activity. The observed toxicity of eel extracts was compared to that predicted from their concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PnCDF) and 3,3',4,4',5-pentachlorobiphenyl (PCB126), using the relative potencies (RePs) of these DLCs previously estimated for *F. heteroclitus* ELS (Rigaud et al., 2013, 2014).

3.4 Materials and methods

3.4.1 Eel collection

A total of 15 large-sized (>80 cm) yellow or silver (sexually maturing) female American eels (*Anguilla rostrata*), 5 per sampling year (1988, 1998 and 2008), were used in this study. These 15 eels were a subsample of 30 eels (10 per sampling year) chemically characterized for POPs in Byer (2013). Eels sampled in July 1988 and June 1998 were collected near Main Duck Island (43°55.76'N, 76°36.11'E), LO, and archived in freezers at -20°C. Additional eels were collected in June 2008 at the Prince Edward Bay of the eastern LO (43°57.01'N, 76°58.01'E), and were stored frozen at -80°C at the Fish Contaminants Laboratory of Environment Canada (Burlington, ON, Canada).

Eel body masses (M) and lengths (L) were measured, and their condition factors (CF)

were calculated ($CF = [M(g)/L^3(cm)] * 100$). Age was determined from transverse sections of sagittal otoliths (360 ± 30 μm) and acetate replicas, deciphered by a Calcified Structure Age-Growth data Extraction Software (CSAGES) and a standardized Calcified Structure Age Interpretation System (CSAIS) (Casselman and Scott, 2000). Age interpretation was performed by counting annuli, using a validated technique (Casselman, 1987) involving both known-age and partly known-age eels. For each sampling year, the 5 eels were selected for chemical analysis and toxicity testing based on their age, so that the average approximated 20 years.

3.4.2 Eel extracts and TCDD solution preparation

TCDD, in acetone (>98% purity), was purchased from Wellington Laboratories (Guelph, ON, Canada). Triolein was obtained from Sigma-Aldrich (St-Louis, MO, USA): triolein is an unsaturated triglyceride previously used as a vehicle solvent in several fish embryotoxicity studies (Walker et al., 1996b). A stock solution of TCDD in triolein was prepared and its concentration (15 ng μL^{-1}) was validated as described in Rigaud et al. (2013).

Whole eel homogenates were prepared in accordance with standard lab practices (Byer et al., 2013b; Kiriluk et al., 1997). Eel tissue extracts were prepared specifically for embryotoxicity assays from approximately 200 g of homogenate per eel. Extractions were performed by the Analytical Service Unit (ASU) of Queen's University (Kingston, ON, Canada) following the protocol described in Kennedy (2010), using dichloromethane and Soxhlet columns. Supplementary lipid removal was performed at the Maurice Lamontagne Institute (Mont-Joli, QC, Canada) following a two-step procedure. The first step consisted of an acidic treatment using sulphuric acid (H_2SO_4) followed by a filtration on sodium sulphate (Na_2SO_4) bound to glass wool. The second step consisted of a gel permeation chromatography (GPC) using Biobeads® S-X3 support (Bio-Rad Laboratories, Mississauga, ON, Canada). Stock solutions of eel extracts (N = 15, 5 per sampling year) were obtained after GPC by evaporating solvent and by adding 40 μL of triolein to the residual complex organic mixture extracted

from 200 g of each individual eel. The concentration of eel extracts in terms of eel tissue equivalent (EEQ) in stock solutions was 5 mg nL^{-1} (200 g of tissue extracted into 40 µL).

3.4.3 Chemical analyses

The purity of triolein was verified using high resolution gas chromatography-mass spectrometry (GC-MS) as described in Rigaud et al. (2013). All fifteen eel homogenates were chemically characterized for PCDD/Fs, PCBs, PBDEs, and OCPs (Byer, 2013; Byer et al., 2013a,b). Measured OCPs were dichlorodiphenyltrichloroethane and its metabolites (DDTs), heptachlor, heptachlor epoxide (isomer B), oxychlordane, α and γ-chlordane, cis and trans nonachlor (CHLs), tris(4-chlorophenyl)methanol and tris(4-chlorophenyl)methane (TCPM and TCPMe, TCPs), hexachlorocyclohexanes (HCHs), hexachlorobenzene (HCB), mirex and dieldrin.

Additional chemical analyses were performed on 9 of 15 eel extracts stock solutions in triolein (3 per sampling year, randomly selected) to assess the impact of their specific preparation (supplementary lipid removal and solvent exchange from dichloromethane to triolein, see section 3.4.2 for details) on their original chemical composition determined by Byer (2013). The same surrogates used for chemical analyses of PCDDs, PCDFs and dioxin-like PCBs in Byer et al. (2013a) and others PCBs, PBDEs and OCPs in Byer et al. (2013b) were added to these 9 eel extracts. Triolein was eliminated from these extracts by acidic treatment using sulphuric acid (H_2SO_4) and the extracts were characterized for DLCs (PCDDs, PCDFs and dioxin-like PCBs) and others compounds (others PCBs and OCPs) following procedures described in Byer et al. (2013a) and Byer et al. (2013b), respectively.

3.4.4 Model species: spawner and egg care

A total of 300 mummichog (*Fundulus heteroclitus*) adults (200 females and 100 males) were collected in May 2010 in Horton's Creek (Miramichi, NB, Canada), a low contaminated

area in terms of POPs (Couillard and Nellis, 1999). Fish were kept at the Maurice Lamontagne Institute in two 420 L fiberglass tanks filled with flowing 25‰ salt water (St. Lawrence Estuary water filtered on 0.8-1.2 mm-thick silicate sand), under controlled photoperiod (16 h:8 h). Water temperature was raised from approximately 10°C to 19°C to stimulate mummichog reproduction (Boyd and Simmonds, 1974). Fish were fed *ad libitum* with pelleted cichlid food (Nutrafin®, Montréal, QC, Canada), 3-mm extruded fish feed (Skretting®, Vancouver, BC, Canada), dried shrimps and *Tubifex* worms. Artificial spawning substrates were placed in the tanks during the afternoon and eggs were collected the next morning. Normal and fertilized eggs between stage 9 and 11 (Armstrong and Child, 1965) were kept for bioassays.

3.4.5 Embryo exposure, incubation and hatching

Embryo exposures were performed on day 0 post-fertilization (DPF). Embryos were exposed by nano-injection of the desired triolein solution: embryos were nested in custom made agarose support plates and injected with an Eppendorf FemtoJet® express microinjector (Eppendorf® Canada, Mississauga, ON, Canada) coupled to a stereo microscope, using quartz needles filled with the desired solution (Rigaud et al., 2013). The injected volume was set at 10 nL per embryo in all cases. Preliminary experiments involving embryos injected with 10 nL of pure triolein and non-injected controls confirmed that injected volumes up to 10 nL had no effects on survival and normal development of control embryos and larvae (data not shown).

Single dose experiments were first conducted to compare the embryotoxicity of the 15 eel extracts (5 per sampling year). Embryos (N = 35 per extract) received an IVi injection of a single dose of eel extract (10 nL). This volume contains POPs extracted from 50 mg of eel tissue, i.e. 50 mg eel tissue equivalent (EEQ). Because the average wet mummichog embryo mass is 5 mg, the corresponding nominal exposure dose was 10 EEQ g^{-1} ww (wet weight), i.e. ten times the concentration of POPs found in eel tissue. In each experiment at least one

extract per sampling year was included.

Three dose-response experiments were performed using three randomly selected eel extracts (one per sampling year): embryos were exposed to increasing doses (N = 35 embryos per dose) of each extract: 0.5, 1, 2.5, 5 and 10 EEQ g^{-1} ww, prepared by serial dilutions of eel extract stock solutions in triolein. Non-injected and injected controls were included in each experiment (N = 35 for each control group). Injected controls were dosed with triolein cleaned up with the same two-step procedure mentioned in section 3.4.2 (i.e. acidic treatment and GPC). Positive controls injected with 160 pg g^{-1} ww of TCDD (N = 20) (Rigaud et al., 2014) were included in each single dose or dose-response experiment to ensure that the experimental protocols were actually capable of detecting effects of dioxin-like compounds and to assess possible temporal variations in sensitivity of the embryos to a reference chemical.

After injection, embryos were randomly distributed and incubated in 24-well Costar® polystyrene microplates (Corning Inc. Life Sciences, Lowell, MA, USA) for 13 days at 22.5°C, under natural photoperiod (14 h:10 h). Embryos were incubated individually on moist filter paper and monitored daily for mortality and malformations. Hatching was triggered after 13 DPF by adding 25‰ salt water. After 1 h, all embryos were transferred individually into 20 mL glass vials filled with 15 mL of 25‰ salt water until 16 DPF. Hatching success and mortality were monitored daily between 13 and 16 DPF during water renewal. Larvae were not fed during incubation. Salt water used for embryos and larvae incubation was from the same source as that used for spawners (see section 3.4.4), but was additionally filtered (0.1 µm pore size) and UV-sterilized.

3.4.6 Locomotor activity

For each experiment, the basal locomotor activity of larvae (N = 8-12 per treatment, excluding the positive control group) and their reaction to light was recorded during three successive periods of 5 min using a Zebrabox® (ViewPoint Life Sciences Inc., Montréal, QC,

Canada) (Rigaud et al., 2013). The first and third periods were recorded in the dark, under infrared light, while the second period was recorded under white light to monitor larval reactions to a visual stimulus (MacPhail et al., 2009). Data obtained from the Videotrack® software were expressed for each individual larva as three variables: rate of travel (average swimming speed over each 5 min period, mm s^{-1}), active swimming speed (average swimming speed over each 5 min period, but during active time only, mm s^{-1}) and inactivity (percent of the time the larva spent immobile). When white light was switched on after the first 5 min period in the dark, mummichog larvae typically stopped moving for few seconds and then alternated short movements and resting periods. The number and the duration of the resting periods (considering only the periods of immobility lasting at least 2 s) were counted. The first resting period which occurred just after the light was turned on was defined as the recovery latency period (Rigaud et al., 2013).

3.4.7 Prey capture ability and efficiency

After the locomotor activity assay, one larva from each treatment (excluding the non-injected control group and the positive control group) was randomly disposed in one of 6 adjacent wells of eight or twelve 48-well Costar® polystyrene microplates (N = 8-12 larvae per treatment). Microplates were prepared in advance by adding 20 *Artemia fransciscana* nauplii in each of the 6 adjacent wells (Gahtan et al., 2005). Larval predation on *Artemia* was recorded during 5 min using a digital camera (Canon VIXIA HV30 HD Camcorder®, Canon Canada Inc., ON, Canada) under fluorescent light. Video analysis was performed to count *a posteriori* the number of *Artemia* captured and the number of feeding strikes each 30 s during the 5 min assay. The linear relationships between the number of *Artemia* in the wells and time (min, log-transformed) were assessed by least-square regression for each larva. The rate of decline of counted *Artemia* was used as an index of prey capture ability, and the number of *Artemia* captured per feeding strike was used as an index of prey capture efficiency (Couillard et al., 2011; Rigaud et al., 2013).

3.4.8 Morphometry

At the end of the exposure, larvae were observed individually and blindly (i.e. without knowing the treatment) under a stereo microscope coupled to an ocular micrometer, and body length was measured. Malformations were recorded, especially craniofacial deformities reported in the embryos of various fish species exposed to DLCs, including *F. heteroclitus* (Elonen et al., 1998; Hill et al., 2004a; Rigaud et al., 2013). Cranial morphology of larvae was scored according to the severity of the deformity: 0 for normal larva, 1 for larva with an intermediate craniofacial deformity and 2 for severe craniofacial deformity (fig. 16). The intermediate craniofacial deformity was characterized by a shortened and flattened snout compared to normal, but was still clearly visible in lateral view. The severe craniofacial deformity was characterized by an extremely shortened snout, which was almost hidden behind the ocular globes when the larva was observed in lateral view. Other deformities typical of the blue sac disease (BSD) syndrome, i.e. edemas or hemorrhages (Spitsbergen et al., 1991), were also recorded.

3.4.9 EROD activity measurement

Fifteen unfed larvae per treatment (N = 3 pools of 5 larvae) were frozen in liquid nitrogen and kept at -80°C at the end of the measurement, until EROD activity analyses. EROD activity was measured in homogenates of 5 larvae (Rigaud et al., 2013) using an adaptation of a microplate spectrofluorimetric method (Fragoso et al., 1998), and expressed as pmoles resorufin min^{-1} mg^{-1} protein.

3.4.10 Bioassay- and chemically derived TCDD-TEQ calculations

The toxic potency of complex mixtures of DLCs should be predictable on the basis of their TCDD toxic equivalents (TCDD-TEQs), i.e. the sum of each DLC concentration

times its respective toxic equivalent factor (TEF, i.e. the relative potency of each DLC versus TCDD) (van Zorge et al., 1989). World Health Organization (WHO) reference TEFs for fish are based on embryolethality studies of trout species exposed to single DLCs by IVi injection (Van den Berg et al., 1998).

Chemical analyses data for eel extracts (see section 3.4.3) were compared to those of eel homogenates presented in Byer (2013). Concentrations were compared among homogenates and extracts for each class of POP and for four selected individual DLCs: TCDD, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PnCDD), 2,3,4,7,8-PnCDF and PCB126. According to Byer et al. (2013a), these DLCs are the most important contributors to total TCDD-TEQs (calculated using WHO fish TEFs) in eel homogenates. The average difference in terms of concentration was estimated for each class of POP and for selected DLCs, and was expressed as percentages (%).

Bioassay- and chemically derived TCDD-TEQs for each dose of eel extract in dose-response experiments were estimated using a similar approach to that described in Rigaud et al. (2014). Bioassay-derived TCDD-TEQs were calculated for each eel extract dose using observed EROD induction for each dose and regression parameters of TCDD for EROD activity calculated in Rigaud et al. (2013). Chemically derived TCDD-TEQs were calculated for the 1 EEQ g^{-1} ww dose from the concentration of TCDD, 2,3,4,7,8-PnCDF and PCB126 in eel homogenates times their respective relative potency (ReP) to TCDD (1, 2.40 and 0.71, respectively) for EROD induction in *F. heteroclitus* larva (Rigaud et al., 2013, 2014).Concentrations for these three DLCs were corrected prior to TCDD-TEQ calculations on the basis of their respective mean losses (expressed in %) between eel homogenates and extracts. Chemically derived TCDD-TEQs for other doses (0.5, 2.5, 5 and 10 EEQ g^{-1} ww) were derived from the concentration calculated for the 1 EEQ g^{-1} ww dose.

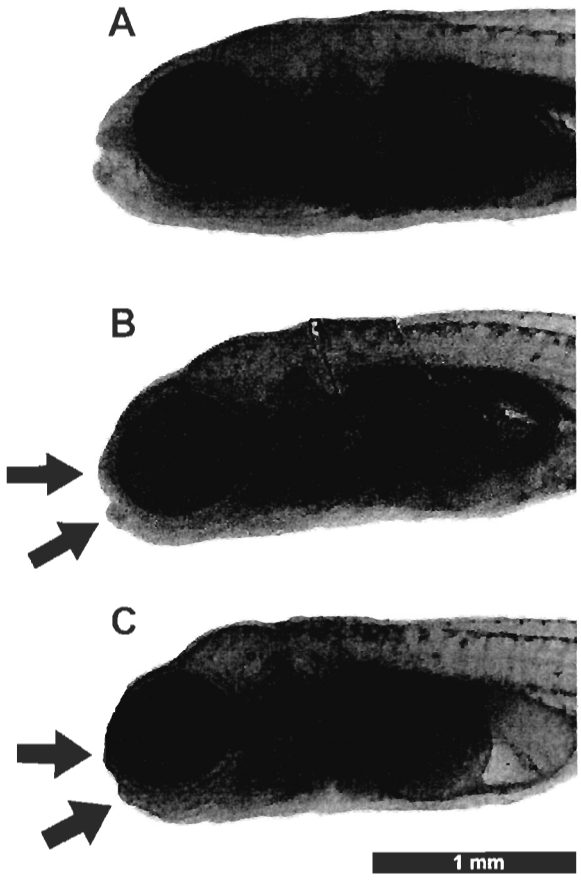

Figure 16: Heads of 16 DPF *F. heteroclitus*: (A) control larva injected with pure triolein with normally developed jaws, (B) larva injected with a 1998 eel extract (10 EEQ g^{-1} ww) showing an intermediate craniofacial deformity and (C) larva injected with a 1988 eel extract (10 EEQ g^{-1} ww) showing a severe craniofacial deformity. Atrophied jaws are indicated by arrows. See description in section 3.4.8.

3.4.11 Data processing and statistical analyses

R 3.0.0 (The R Foundation for Statistical Computing) was used for statistical analyses at $\alpha = 0.05$. Consistency across experiments was assessed using TCDD positive control groups (see section 3.5.6 for details) and all data from single dose experiments were pooled to compare toxicity among sampling years. Body length and EROD activity data were expressed as percentages (%) relative to respective injected controls in single-dose experiments. Comparisons among treatments (i.e. injected, non-injected controls and sampling years) were performed using a non-parametric Kruskal-Wallis test (KW) followed, when significant, by an ANOVA on ranked values and a Tukey's studentized range test for multiple comparisons (Conover and Iman, 1981). For dose-response experiments, log-transformed data for EROD activity were normally distributed and compared among treatments with a one-way ANOVA followed when significant by a Tukey's multiple comparisons test. Data expressed as proportions of larvae (mortality, hatching success or prevalence of deformities) were compared to controls using the Fisher's exact test (FE). The influence of the presence or absence of craniofacial deformities (fig. 16) on body length, prey capture ability and efficiency was assessed: for treatments (in single dose experiments) or doses of eel extracts (in dose-response experiments) showing a significant increase of the prevalence of craniofacial deformities, these responses were compared between impacted and non-impacted individuals within treatments or doses using the Mann-Whitney U test (MW).

Dose-response relationships for EROD activity and prey capture ability were assessed by least square regression. Prior verification of the homoscedasticity and normal distribution of residuals was performed using the White and the Shapiro-Wilk tests, respectively. Data were expressed as percentages relative to triolein controls and log-transformed before regression analysis. For significant dose-response relationships, homogeneity of slopes was assessed. After confirmation of the homogeneity of slopes, intercepts of the dose-response relationships were compared among sampling years using ANCOVA followed by a Tukey's multiple comparisons test when significant. Significant dose-response relationships of eel

extracts for EROD activity or prey capture ability were also individually compared using the same procedure to the TCDD dose-response relationships obtained in Rigaud et al. (2013).

3.5 Results

3.5.1 Eel biological characteristics

Table 13 summarizes age, body length, mass, lipid content and condition factor data of eels for all sampling years. Eels collected in 2008 were longer and had lower lipid content and condition factor compared to those from 1988 and 1998 (KW, $p \leq 0.05$). Eels from 2008 were also heavier compared to those from 1998 (KW, $p \leq 0.05$), but not 1988. As expected since it was a criterion for selection, age of selected eels did not differ among sampling years (KW, $p > 0.05$).

3.5.2 Chemical analyses

Quality assurance and quality control for chemicals analyses of eel homogenates were detailed in Byer (2013). Replicate samples had mean ± SD coefficient of variation (CV) of 22 ± 20% and 14 ± 16% for PCDD/Fs and dioxin-like PCBs, respectively. The mean ± SD CV for reference material (CARP-2, National Research Council of Canada, Ottawa, ON, Canada) was 12 ± 8% for other PCBs, 22 ± 16% for OCPs and 17 ± 21% for PBDEs, suggesting a fairly high degree of reproducibility. The mean ± SD recovery for spiked lab blanks were 98 ± 12% for other PCBs, 97 ± 15% for OCPs and 110 ± 17% for PBDEs.

Concentrations of each class of POP (ΣPCDD/Fs, ΣPCBs, ΣPBDEs and OCPs) in the subsample of homogenates selected for embryotoxicity assays were similar in 1988 and 1998 eels, but lower in 2008 eels (KW, $p \leq 0.05$) (table 14). Concentrations of TCDD, 1,2,3,7,8-PnCDD and 2,3,4,7,8-PnCDF were similar in 1988 and 1998 eels but lower in 2008 eels (KW,

Table 13: Body mass, length, age, lipid content and condition factor of eels collected in LO in 1988, 1998 and 2008 and used in the present study. Values are expressed as median (Q1 - Q3). Different letters indicate a significant difference (KW, $p \leq 0.05$) between sampling years.

Sampling year	*N*	Sex	Age (years)	Body length (cm)	Body mass (kg)	Lipid content (%)	Condition factor (g per cm^3)
1988	5	Female	21.0 (19.0 - 21.5)	96.6 (91.2 - 102.2) [a]	2.2 (2.0 - 2.5) [ab]	27.1 (26.6 - 29.5) [a]	0.25 (0.23 - 0.27) [a]
1998	5	Female	19.0 (16.5 - 20.5)	84.8 (82.6 - 95.3) [a]	1.9 (1.4 - 2.3) [a]	27.0 (25.9 - 32.8) [a]	0.26 (0.23 - 0.32) [a]
2008	5	Female	22.0 (20.0 - 24.0)	118.0 (108.0 - 123.0) [b]	2.8 (2.4 - 3.3) [b]	23.4 (21.3 - 25.2) [b]	0.17 (0.16 - 0.21) [b]

$p \leq 0.05$) (table 14). For PCB126, however, concentrations differed (KW, $p \leq 0.05$) among all sampling years, with approximately 4-fold higher concentrations in 1988 and 1998 eels compared to 2008 eels (table 14).

Concentrations for each class of POP were generally lower in extracts compared to homogenates (table 14), suggesting chemical loss during the preparation of eel extracts stock solutions into triolein. Losses were estimated at 19.8% for ΣPCDD/Fs, but were generally higher and close to 50% when considering individual selected DLCs contributing the most to total TCDD-TEQs (table 14). For the most potent dioxin-like PCB, PCB126, losses for the 1988, 1998 and 2008 extracts were respectively 43%, 37% and 22%. Losses were also close to 50% for ΣPCBs (table 14). Except DDTs, CHLs and mirex for which losses ranged from 49.9% to 68.7%, all others OCPs (HCHs, TCPs, HCH and dieldrin) were almost absent in eel extracts stock solutions (table 14).

3.5.3 Mortality and hatching success

Mortality rate in single dose experiments did not differ (KW, $p > 0.05$) between injected and non-injected controls, but was more variable for injected controls: 27% (7-47) and 15% (12-19), respectively (median, Q1-Q3). In dose-response experiments, mortality rate did not differ (FE, $p > 0.05$) between injected (6%, 4-11) and non-injected (6%, 3-7) controls (median, Q1-Q3). Mortality was not induced (KW, $p > 0.05$) by any eel extract for any sampling year or dose, or by any TCDD control group (data not shown).

At DPF 16, hatching success in single dose experiments did not differ (KW, $p > 0.05$) between non-injected (93%, 88-97) and injected controls (89%, 87-95) (median, Q1-Q3). Hatching success between DPF 13 and DPF 16 was not altered (KW, $p > 0.05$) by any eel extract or by any TCDD control group (data not shown). In dose-response experiments, no difference (FE, $p > 0.05$) in hatching success was observed between non-injected (83%, 82-91) and injected controls (91%, 88-95) at 16 DPF (median, Q1-Q3). Hatching success was

Table 14: Mean concentrations[1] (± standard deviations) of ΣPCDD/Fs, selected DLCs, ΣPCBs, OCPs and ΣPBDEs in eels homogenates ($N = 5$) and extracts ($N = 3$). The average differences between eel homogenates and extracts used for *F. heteroclitus* embryos exposure are also presented (medians and Q1-Q3) for each class of POP and selected individual dioxin-like compounds. Different letters indicates a significant difference among sampling years for eel homogenates or extracts (KW, $p \leq 0.05$).

	1988		1998		2008		
	Homogenates	Extracts	Homogenates	Extracts	Homogenates	Extracts	Average difference (%)
ΣPCDD/Fs	23.7 ± 10.3 [a]	28.0 ± 0.5	22.2 ± 4.5 [a]	21.4 ± 9.2	8.0 ± 3.7 [b]	5.5 ± 4.4	19.8% (0.0 - 36.4)
Selected DLCs							
TCDD	5.1 ± 0.7 [a]	2.3 ± 0.5	5.7 ± 1.3 [a]	2.5 ± 0.5	1.4 ± 0.7 [b]	N.D.[2]	60.9% (60.7 - 61.3)
1,2,3,7,8-PnCDD	2.0 ± 0.9 [a]	1.4 ± 0.5	1.7 ± 0.6 [a]	1.5 ± 0.7	0.7 ± 0.3 [b]	N.D.	47.4% (20.3 - 56.5)
2,3,4,7,8-PnCDF	8.3 ± 2.5 [a]	5.0 ± 2.6	7.7 ± 0.9 [a]	2.8 ± 0.8	1.7 ± 0.5 [b]	3.1 ± 2.4	46.7% (12.8 - 51.8)
PCB126	492 ± 102 [a]	279 ± 64	370 ± 75 [b]	233 ± 34	99 ± 35 [c]	77 ± 41	45.5% (34.0 - 48.8)
ΣPCBs	1235 ± 395 [a]	745 ± 234 [a]	988 ± 192 [a]	689 ± 211 [ab]	341 ± 212 [b]	216 ± 164 [b]	48.5% (43.5 - 52.5)
OCPs							
ΣDDTs	241 ± 95 [a]	112 ± 13	238 ± 39 [a]	129 ± 16	108 ± 43 [b]	96 ± 45	49.9% (29.5 - 57.8)
ΣCHLs	176 ± 72 [a]	62 ± 20	132 ± 39 [a]	50 ± 7	32 ± 21 [b]	15 ±7	68.7% (66.3 - 70.5)
ΣHCHs	4.3 ± 0.5 [a]	N.D.	4.8 ± 0.3 [a]	N.D.	0.6 ± 0.1 [b]	N.D.	100.0%
ΣTCPs	2.2 ± 0.6 [a]	N.D.	1.5 ± 0.4 [a]	N.D.	0.4 ± 0.2 [b]	N.D.	100.0%
HCB	12.8 ± 4.9 [a]	2.1 ± 0.5 [a]	11.0 ± 3.1 [a]	1.2 ± 0.4 [ab]	3.3 ± 1.3 [b]	0.6 ± 0.4 [b]	88.2% (87.0 - 91.3)
Mirex	104 ± 34 [a]	34 ± 14	87 ± 26 [a]	44 ± 4	21 ± 14 [b]	15 ± 10	54.7% (34.7 - 61.7)
Dieldrin	51 ± 11 [a]	N.D.	47 ± 14 [a]	N.D.	13 ± 5 [b]	N.D.	100.0%
ΣPBDEs	71 ± 5	N.M.[3]	68 ± 11	N.M.	24 ± 22	N.M.	N.A.[4]

Note: data presented here is for a subsample of 15 of 30 eels (5 per sampling year) originally chemically characterized for POPs in Byer (2013).

[1] Concentrations for ΣPCDD/Fs and selected dioxin-like compounds are in pg g^{-1} ww. Concentrations for other compounds are in ng g^{-1} ww.

[2] Not detected.

[3] Not measured.

[4] Not applicable.

only significantly reduced by the two highest exposure doses (5 and 10 EEQ g^{-1} ww) of the 1988 extract at DPH0 and DPH1, but not at DPH2 and 3 (data not shown).

3.5.4 Body length and deformities

At DPF 16, body length did not differ between non-injected controls, triolein injected controls or TCDD positive controls in all experiments (data not shown). At 10 EEQ g^{-1} ww, body length of mummichog larvae was reduced by exposure to 1988 or 1998 eel extracts (KW, $p \leq 0.05$), but not to 2008 eel extracts (fig. 17). In dose-response experiments, body length was reduced by exposure to 1988 and 1998 extracts at ≥5 and ≥2.5 EEQ g^{-1} ww, respectively (KW, $p \leq 0.05$), but not by the 2008 extracts (fig. 18).

Edemas or hemorrhages were very sporadic and not significantly induced by any treatment in any experiment. Craniofacial deformities were the only kind of malformations regularly observed (fig. 16). These deformities were not observed in non-injected and injected controls (data not shown). In single dose-experiments, severe craniofacial deformities were mainly observed following exposure to the 1988 extracts, with median (Q1-Q3) prevalence of 11% (0-42), but this was not significant compared to injected controls (KW, $p > 0.05$). However, when all craniofacial deformities were considered (intermediate and severe), an increased prevalence was induced (KW, $p \leq 0.05$) by 1988 (72%, 59-89) and 1998 (74%, 55-81) extracts, but not by the 2008 extracts (7%, 3-17) or TCDD (3%, 0-20) for positive controls (median, Q1-Q3). In dose-response experiments, the prevalence of severe craniofacial deformities was induced at ≥5 and 10 EEQ g^{-1} ww by the 1988 and 1998 extracts, respectively (FE, $p \leq 0.05$). When all craniofacial deformities (intermediate and severe) were considered, an increased prevalence was induced at ≥2.5 and 10 EEQ g^{-1} ww by the 1988 and 1998 extracts, respectively (FE, $p \leq 0.05$).

The influence of the presence or absence of craniofacial deformities on body length, prey capture ability and efficiency was studied for the treatments or doses where the preva-

lence of these malformations was significantly increased. In single dose experiments, larvae exposed to the 1988 eel extracts and showing a craniofacial deformity were significantly smaller compared to unaffected larvae (MW, $p \leq 0.05$), while no significant difference was observed between affected and unaffected larvae for the 1998 eel extracts. In dose-response experiments, at the highest dose (10 EEQ g^{-1} ww) of the 1998 eel extract, affected larvae were significantly smaller compared to unaffected ones (MW, $p \leq 0.05$). However, for the 1988 eel extract, no significant difference was observed between affected and unaffected larvae at doses where the prevalence of craniofacial deformities was significantly increased (i.e. at $\geq$2.5 EEQ g^{-1} ww). Regarding the larvae used for the behavioral tests, individuals exposed to the 1988 eel extracts in single dose experiments and showing a craniofacial deformity had a significantly lower prey capture ability and efficiency compared to unaffected individuals (MW, $p \leq 0.05$). It was not the case with the 1998 eel extracts in single dose experiments, or at any dose in dose-response experiments. However, in this latter case, the influence of the presence or absence of craniofacial deformities was not testable for the two highest doses (5 and 10 EEQ g^{-1} ww) of the 1988 eel extract because all the larvae used for behavioral test were affected.

3.5.5 Locomotor activity and predatory capacity

None of the eel extracts at any dose and in any experiments affected basal locomotor activity or reaction to light (data not shown). In single dose experiments, both prey capture ability and efficiency of mummichog larvae were reduced by exposure to the 1988 and 1998 eel extracts (KW, $p \leq 0.05$) (fig. 17). The 1988 extracts were more potent to reduce prey capture ability or efficiency than the 1998 extracts (fig. 17). Some larvae exposed to the 1988 or 1998 eel extracts captured a relatively low number of *Artemia* despite attempting numerous feeding strikes (fig. 19). In dose-response experiments, only two larvae exposed to the highest dose of the 1988 (10 EEQ g^{-1} ww) extract showed a similar tendency (data not shown). Exposure to the 1988 extract reduced (KW, $p \leq 0.05$) both prey capture ability and

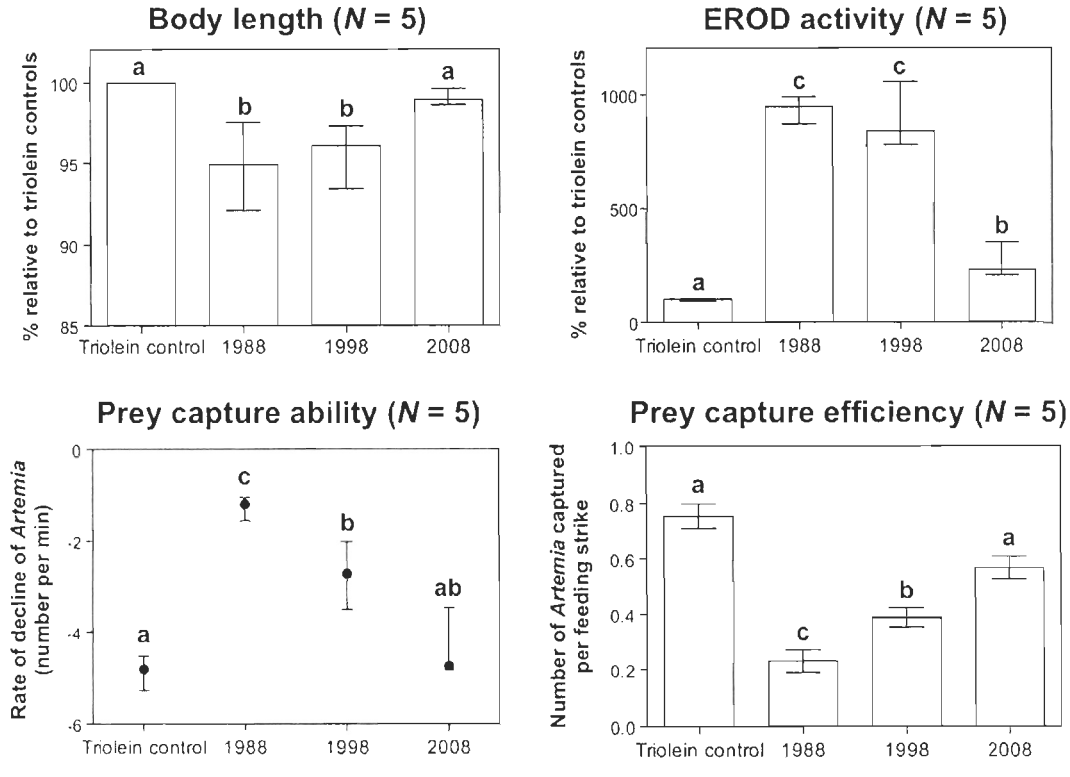

Figure 17: Effect of single-dose exposure (10 EEQ g^{-1} ww) of 16 DPF *F. heteroclitus* larvae to eel extracts on body length, EROD activity, prey capture ability and efficiency. Different letters indicate a significant difference (KW, $p \leq 0.05$) among sampling years or between sampling years and triolein injected controls. Values are expressed as median and interquartile range. The non-injected control group is not shown. $N = 5$ eel extracts per sampling year.

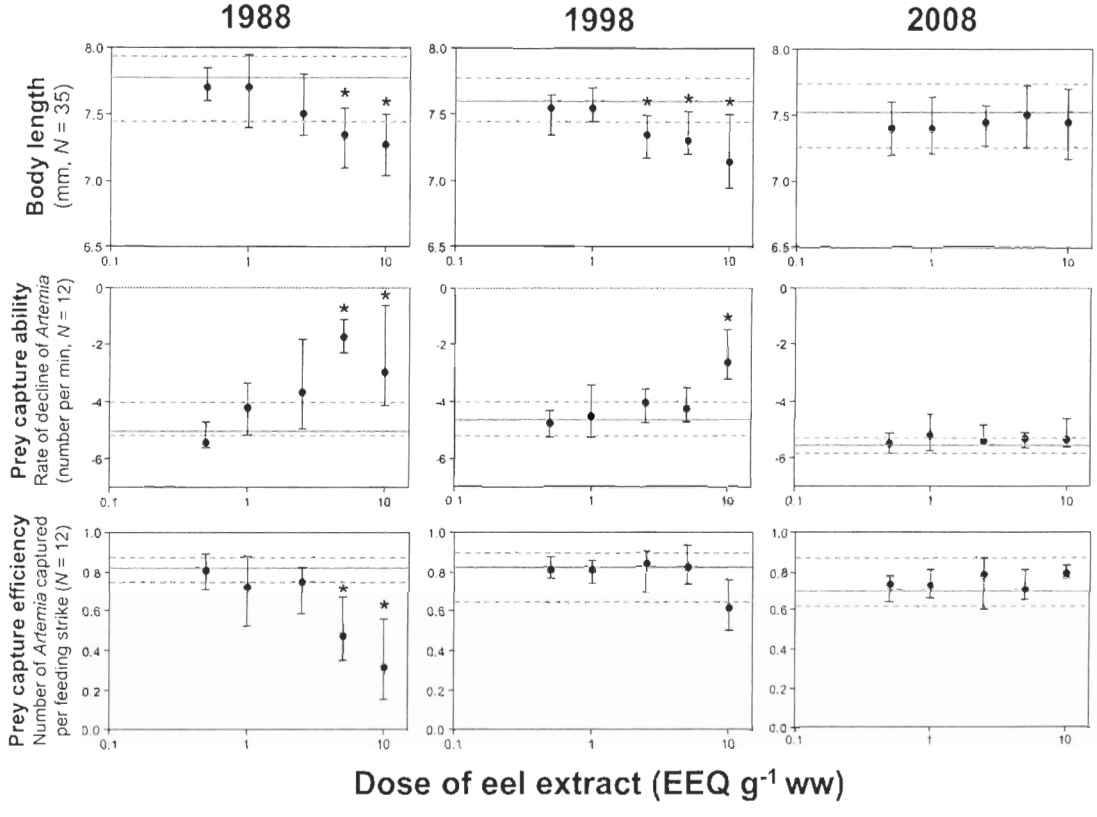

Figure 18: Effect of different doses (0.5 to 10 EEQ g^{-1} ww) of three eel extracts (one per sampling year) on body length, prey capture ability and efficiency of 16 DPF *F. heteroclitus* larvae. Values are expressed as median (dots) and interquartile range (vertical bars, Q1-Q3). Solid and dotted horizontal lines represent median value and interquartile range for the triolein control group. The non-injected control group is not shown. * Indicates a significant difference compared to the triolein control group (KW, $p \leq 0.05$).

efficiency at ≥5 EEQ g^{-1} ww (fig. 18). Exposure to the 1998 extract significantly reduced prey capture ability at 10 EEQ g^{-1} ww, while prey capture efficiency tended to be reduced at the same dose, but it was not significant (fig. 18). Dose-response exposure to the 2008 extract did not reduce prey capture ability or efficiency (fig. 18). In all experiments, none of the tested eel extracts at any dose altered the number of feeding strikes attempted by larvae during prey capture assays (data not shown).

Prey capture ability decreased with dose for the 1988 and 1998 eel extracts, but not for the 2008 extract (table 15). Comparison of the dose-response relationships between 1988 and 1998 revealed that slopes were homogenous ($p = 0.07$), but the intercept for the 1988 extract was higher compared to the 1998 extract (ANCOVA, $p \leq 0.05$, table 15). The slope of the 1988 extract was significantly higher compared to TCDD ($p \leq 0.05$), while no significant difference was observed between slopes for the 1998 extract and TCDD ($p \leq 0.05$, table 15). In this last case, the intercept of the dose-response relationship of TCDD was significantly higher compared to that of the 1998 extract (ANCOVA, $p \leq 0.05$, table 15).

3.5.6 EROD activity

As expected, EROD activity did not differ between injected and non-injected controls in all experiments (data not shown). Mean ± SD EROD activity of the TCDD positive control groups were 2.31 ± 0.80 and 1.86 ± 0.55 pmoles resorufin min^{-1} mg^{-1} protein in single dose ($N = 4$) and dose-response experiments ($N = 3$), respectively. EROD activity in the TCDD positive control groups did not differ among experiments (ANOVA, $p > 0.05$, data not shown), indicating consistent dosing method and embryo sensitivity among experiments.

In single dose experiments, EROD activity was induced (KW, $p \leq 0.05$) by all eel extracts from all sampling years (fig. 17). However, induction was significantly higher for the 1988 and 1998 extracts compared to the 2008 extracts (fig. 17). EROD induction did not differ between the 1988 and 1998 extracts (fig. 17). In dose-response experiments, the 1988

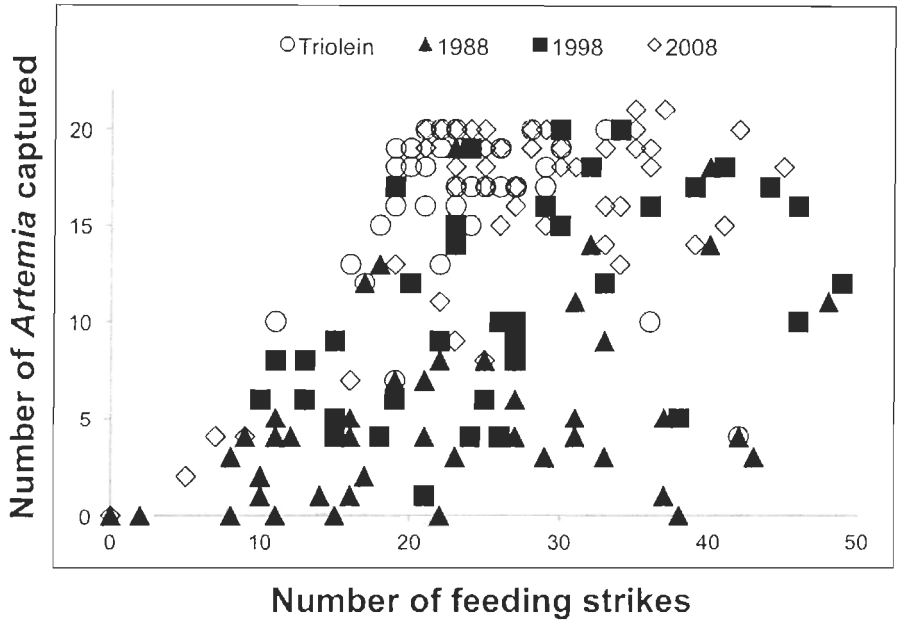

Figure 19: Relationships between the number of *Artemia* captured and the number of feeding strikes for each larva following single-dose exposure (10 EEQ g^{-1} ww) of 16 DPF *F. heteroclitus* larvae to eel extracts.

Table 15: Regression parameters relating the log of the EROD activity or prey capture ability (expressed as percentages relative to injected controls) and the log of the dose for eel extracts and TCDD.

	Regression parameters[1]						ANCOVA vs. TCDD[2]	
	a	*b*	*F*-statistic	DF	R^2	*p*	Slope	Intercept
EROD activity								
Eel extract, 1988	0.33 ± 0.06	2.39 ± 0.04 [a]	29.25	13	0.69	<0.001	<0.01	
Eel extract, 1998	0.41 ± 0.09	2.35 ± 0.05 [a]	21.79	13	0.63	<0.001	0.06	<0.001
Eel extract, 2008	0.35 ± 0.07	2.11 ± 0.04 [b]	25.29	13	0.66	<0.001	<0.01	
TCDD[3]	0.62 ± 0.06	1.25 ± 0.13	102.72	9	0.92	<0.001		
Prey capture ability								
Eel extract, 1988	0.57 ± 0.11	1.97 ± 0.07 [a]	25.64	52	0.33	<0.001	<0.01	
Eel extract, 1998	0.32 ± 0.08	1.88 ± 0.05 [b]	16.59	58	0.22	<0.001	0.21	<0.001
Eel extract, 2008	No significant dose-response relationship					0.35		
TCDD[3]	0.19 ± 0.07	2.40 ± 0.14	8.34	35	0.19	<0.01		

[1] Model formula is: Log[y] = *a**Log[x] + *b*, where *a* is the slope, *b* the intercept, x the dose and y is EROD activity or prey capture ability (expressed as percentages relative to injected controls).

[2] Comparison of the parameters (slope and intercept) of the dose-response relationship with the one of TCDD, by ANCOVA. Intercepts were not compared when slopes were significantly differents ($p \leq 0.05$).

[3] Regression parameters for TCDD were obtained from Rigaud et al. (2013).

and 1998 eel extracts increased EROD activity at ≥1 EEQ g^{-1} ww, while the 2008 extract only increased EROD activity at 10 EEQ g^{-1} ww (fig. 20) (ANOVA, $p \leq 0.05$). EROD activity increased with dose for eel extracts from all three sampling years (fig. 20 and table 15). Comparison of the dose-response relationships among sampling years revealed that all three slopes were homogenous ($p > 0.05$), but a significant difference was observed among intercepts (ANCOVA, $p \leq 0.05$): the intercepts of the 1988 and 1998 eel extracts dose-response relationships were similar, but significantly higher compared to the intercept of the 2008 eel extract (table 15). Individual comparison of each dose-response relationship to the one for TCDD calculated in Rigaud et al. (2013) revealed that the slope for TCDD was significantly higher than that of the 1988 or 2008 eel extracts ($p \leq 0.05$, table 15). The slopes for TCDD and the 1998 eel extract were homogeneous, but the p-value was very close to the significance level ($p = 0.06$).

3.5.7 Estimation of bioassay- and chemically derived TCDD-TEQs

Bioassay- and chemically derived TCDD-TEQs calculated for each eel extract dose in dose-response experiments are presented in table 16. Due to its high ReP in *F. heteroclitus* and its relatively high concentrations in eel extracts compared to TCDD and 2,3,4,7,8-PnCDF, the contributions of PCB126 to chemically derived TCDD-TEQs were 94.3%, 91.9% and 93.1% in 1988, 1998 and 2008 eel extracts, respectively (data not shown). For all eel extracts at any sampling year or exposure dose, bioassay-derived TCDD-TEQs were 2 to 10 times lower than chemically derived TCDD-TEQs (table 16). The discrepancy between the bioassay- and the chemically derived TCDD-TEQs generally increased with increasing doses of eel extracts, and was higher for older eel extracts compared to recent ones (table 16).

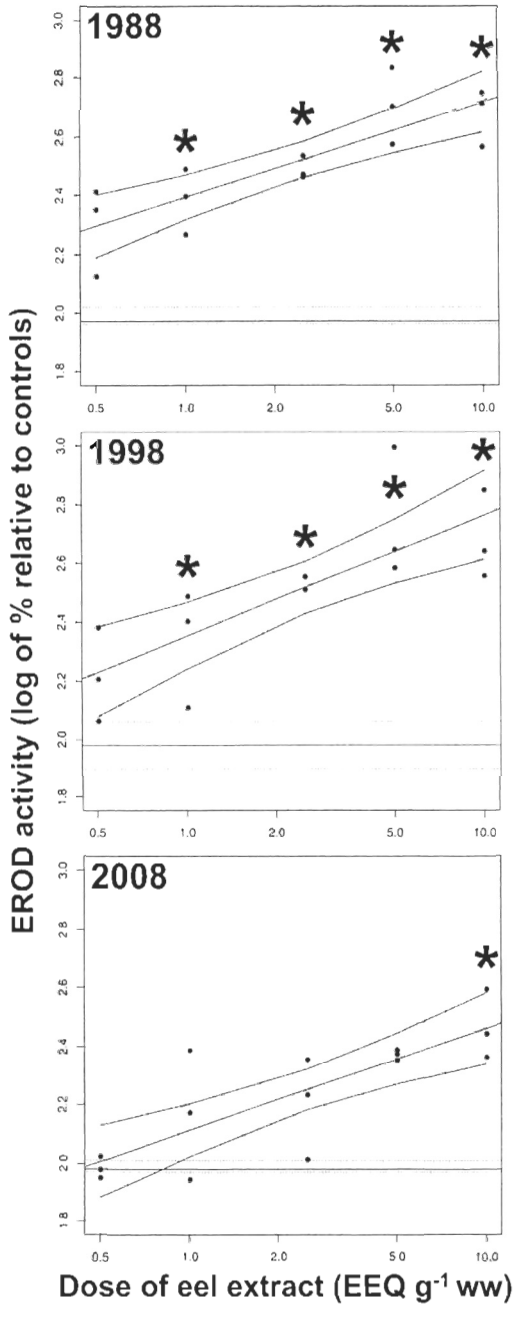

Figure 20: Effect of different doses (0.5 to 10 EEQ g^{-1} ww) of three eel extracts (one per sampling year) on EROD activity (log-transformed, $N = 3$) of 16 DPF *F. heteroclitus* larvae. EROD activity was expressed as percentages relative to triolein controls and log-transformed prior to regression analyses. Solid and dotted horizontal lines represent median value and interquartile range for the triolein control group. The non-injected control group is not shown. * Indicates a significant difference compared to the triolein control group (ANOVA, $p \leq 0.05$).

Table 16: Comparison of bioassay- and chemically derived TCDD-TEQs corresponding to each dose of eel extracts in dose-response experiments.

Sampling year	Exposure dose (EEQ g^{-1} ww)	Bioassay-derived[a] TCDD-TEQs (pg g^{-1} ww)	Chemically derived[b] TCDD-TEQs (pg g^{-1} ww)	Fold difference[c]
1988	0.5	53.4 ± 25.0	111.9	2.1
	1	71.2 ± 28.2	223.7	3.1
	2.5	100.7 ± 15.3	559.3	5.6
	5	236.4 ± 110.9	1118.5	4.7
	10	205.1 ± 65.3	2237.0	10.9
1998	0.5	40.6 ± 23.7	74.6	1.8
	1	64.7 ± 37.5	149.1	2.3
	2.5	119.5 ± 11.2	372.9	3.1
	5	322.7 ± 282.8	745.7	2.3
	10	226.2 ± 131.8	1491.5	6.6
2008	0.5	15.3 ± 2.1	18.5	1.2
	1	37.0 ± 2.9	37.1	1.0
	2.5	38.4 ± 21.6	92.6	2.4
	5	64.2 ± 4.3	185.3	2.9
	10	97.6 ± 43.6	370.6	3.8

[a] Bioassay-derived doses in terms of TCDD-TEQs were calculated using observed EROD activity induction following exposure of *F. heteroclitus* embryos to eel extracts in dose-response experiments and regression parameters for TCDD (table 15).

[b] Chemically derived doses in terms of TCDD-TEQs were determined from the concentrations of TCDD, 2,3,4,7,8-PnCDF and PCB126 measured in eel homogenates (see Section 2.10 for details).

[c] Fold difference between bioassay- and chemically derived TCDD-TEQs.

3.6 Discussion

3.6.1 Temporal trends in the embryotoxicity of eel extracts to *F. heteroclitus* embryos

This study has revealed that complex organic mixtures extracted from American eels captured in LO between 1988 and 2008 were embryotoxic to developing *F. heteroclitus* embryos and larvae, and that the toxic potency of these extracts varied among years. The 1988 and 1998 eel extracts were the most toxic and caused a pattern of sublethal embryotoxic responses similar to those previously reported in *F. heteroclitus* embryos exposed to single DLCs: stunted growth, craniofacial deformities, induction of EROD activity, and reduced predatory capacities (Rigaud et al., 2013, 2014). The pattern of behavioral responses observed with the eel extracts was typical of that previously described in *F. heteroclitus* injected with DLCs: the reduction in prey capture ability was associated with a reduced prey capture efficiency, without alteration of locomotor activity and not necessary coupled to craniofacial deformities (Couillard et al., 2011; Rigaud et al., 2013, 2014). The extracts did not produce any other types of malformations (such as vertebral malformations, for example) than those observed in previous studies in *F. heteroclitus* exposed to environmentally realistic doses of DLCs.

The potency of the extracts to stunt growth, reduce predatory capacities and induce EROD activity declined over time. The 1988 and 1998 eel extracts were the most toxic, altering prey capture ability at ≥5 and 10 EEQ g^{-1} ww respectively, whereas the 2008 extracts did not affect this response. EROD activity was the most sensitive endpoint with significant induction at ≥1 EEQ g^{-1} ww for the 1988 and 1998 eel extracts and at 10 EEQ g^{-1} ww for the 2008 extract. The results of these embryotoxicity bioassays are consistent with temporal declining trends in the concentrations of POPs accumulated in LO eel fat, with higher concentrations for all POPs analyzed in 1988 and 1998 compared to 2008 (table 14) (Byer, 2013).

3.6.2 Predictability of the toxic potency of eel extracts

This study demonstrated that complex mixtures of organic compounds in eel extracts were less toxic to developing *F. heteroclitus* than predicted by their concentration of PCB126 alone. Chemically derived TCDD-TEQs based on TCDD, 2,3,4,7,8-PnCDF and PCB126 concentrations and calculated using species-specific RePs (Rigaud et al., 2013, 2014) overestimated the potency of eel extracts to induce EROD activity in *F. heteroclitus* larvae. Chemically derived TCDD-TEQs were 2 to 10 times higher than bioassay-derived TCDD-TEQs (based on EROD activity) at any dose and for any sampling year, and these differences increased with increasing doses of eel extracts (table 16). Lower bioassay-derived TCDD-TEQs compared to chemically derived TCDD-TEQs were also observed for *F. heteroclitus* ELS treated with IVi injection of sublethal doses of a complex mixture of PCBs, Aroclor 1254 (Rigaud et al., 2014). Interactions among dioxin-like and non-dioxin-like PCBs including competition for the AHR and altered tissue distribution leading to lower concentrations at target sites were proposed as possible mechanisms for the observed reduction in the toxic potency of DLCs in this complex mixture (Rigaud et al., 2014). It may also be the case for eel extracts as they represent organic mixtures even more complex than Aroclor 1254, containing a wider range of DLCs, as most PCDD congeners are not detected in Aroclor 1254 mixtures (Johnson et al., 2008). As well, some classes of POPs measured in the present study are not present in Aroclor mixtures (i.e. PBDEs and OCPs). Eels may also bioaccumulate emerging pollutants of concern not measured in the present study, such as perfluorooctane sulfonic acid (PFOS) (Hoff et al., 2005), various brominated flame retardants (BFRs) used as alternatives to PBDEs (Byer et al., in press; Sühring et al., 2013), polybrominated dibenzo-*p*-dioxins (PBDDs) and polybrominated dibenzofurans (PBDFs) (van den Berg et al., 2013) that could also possibly alter the toxicodynamics and toxicokinetics of potent DLCs.

As the effects observed were typical of those induced by DLCs and were less severe than those predicted based on the extracts concentrations of PCB126 alone (Rigaud et al., 2013, 2014), it suggests that other POPs (non-dioxin-like PCBs, PBDEs, OCPs and unmea-

sured POPs) were not important agonistic contributors to the observed toxicity. Neurobehavioral disturbances that could have been induced by a variety of non-DLCs POPs would presumably have been captured by our behavioral assays measuring locomotor activity, reaction to light and prey capture ability. Regarding non-dioxin-like PCBs, it is consistent with an earlier study, which showed that IVi injection of sublethal and environmentally relevant doses of PCB52 and PCB110 had no effects on survival, growth, malformations, locomotor activity or prey capture ability of *F. heteroclitus* ELS (Rigaud et al., 2014). Some legacy OCPs such as DDT, lindane (also known as hexachlorocyclohexane, HCH) or chlordane are known to cause neurotoxicity and/or behavioral disruption in adult or developing fish (Anderson and Peterson, 1969; Anderson and Prins, 1970; Bhattacharjee and Das, 2013; Gooch et al., 1990; Tiedeken and Ramsdell, 2009). In the present study, many OCPs were lost during eel extract preparation (table 14). This is likely due to the acidic treatment used as a first step to remove residual lipids before the transfer of eel extracts into triolein: some OCPs are known to be destroyed following cleaning procedures involving sulphuric acid (Chung and Chen, 2011). Commercial mixtures of PBDEs (DE-71) or individual congeners (mainly BDE-47 and BDE-209) induced neurotoxicity, altered behavior and/or reduced mobility during ELS of *F. heteroclitus* (Timme-Laragy et al., 2006) and *D. rerio* (Chen et al., 2013; He et al., 2011; McClain et al., 2012; Usenko et al., 2011; Zhao et al., 2014). However, in these studies, exposures of embryos to PBDEs were performed using aqueous exposure and/or at environmentally unrealistic doses reaching thousands of ng g^{-1} ww: concentration of ΣPBDEs in zebrafish larvae ranged between 1000 and 3000 ng g^{-1} ww in studies where internal concentration were measured (Chen et al., 2013; He et al., 2011), which is much higher than the concentrations measured in eel homogenates in the present paper (i.e. 71 ± 5 ng g^{-1} ww for 1988). Moreover, preliminary experiments involving *F. heteroclitus* embryos injected with doses of BDE-47 as high as 5400 ng g^{-1} ww showed no significant effects on survival, growth, basal locomotor activity or prey capture ability of 16 DPF larvae (Rigaud et al., unpublished results).

Chemically derived TCDD-TEQs of eel extracts using WHO reference TEFs for trout

species were calculated by Byer (2013) in order to estimate the toxicity of eel homogenates from chemical characterization data only. This author reported values equal to 15.3 ± 2.8, 13.5 ± 3.1 and 3.5 ± 1.7 pg TCDD-TEQ g^{-1} ww for 1988, 1998 and 2008 eel homogenates, respectively. Assuming that mortality thresholds of trout species (Cook et al., 2003) are applicable to eels, these results suggest that the sublethal effects guideline of 5 pg TCDD-TEQ g^{-1} was exceeded by all 1988 and 1998 eels (Byer, 2013), which is consistent with the results of the present study. The fact that 1988 and 1998 eel extracts pose a risk of toxic effects in phylogenetically distant species increases the weigh of evidence for possible toxic effects of DLCs in early life stage of eels during and prior this time period.

3.6.3 Perspectives

Overall, our results support the hypothesis that contamination of LO with DLCs may have represented a threat for the American eel population through ecologically relevant effects on larval prey capture ability. While concentrations of DLCs have declined in North America, there is evidence that current levels in European eel (*Anguilla anguilla*) tissues remain high, mainly due to elevated concentrations of dioxin-like PCBs (Byer, 2013; Byer et al., 2013a). In this study, the embryotoxicity of extracts from only 5 LO eels per sampling year was assessed, but eels collected in 2008 are a representative sub-sample of a larger sample of LO eels (N = 17) for which concentrations of DLCs have been measured. Measured concentrations of PCB126, the most potent DLC, were 124 ± 86 pg g^{-1} ww in this larger sample of 2008 LO eels (Byer, 2013); whereas, they were 99 ± 35 pg g^{-1} ww in the subsample of eels used for the assays and 77 ± 41 pg g^{-1} in the extracts (this study). Concentrations of DLCs measured in whole body homogenates may underestimate concentrations in the eggs as suggested by a previous study on tissue distribution of PCBs in silver American eel sampled in St. Lawrence Estuary, with gonad/maternal concentration ratios averaging about 1.4 for PCBs (Hodson et al., 1994). European eels sampled in 2009 from Canal Dessel-Schoten, Belgium had concentrations of 304 ± 181 pg g^{-1} ww PCB126 in their tissues (Byer, 2013),

which were of the same order of magnitude as the concentrations measured in American eel sampled in LO in 1988 and 1998 in this study. Further studies coupling chemical analyses and fish embryotoxicity bioassays on extracts prepared from eels captured at other sites in North America and Europe would be useful for risk assessment.

In this study, prey capture ability was altered at concentrations ≥5 EEQ g^{-1} in 1988 whereas EROD activity was induced at ≥1 EEQ g^{-1} in 1988 and 1998 in our surrogate species. Several factors could have contributed to an underestimation of the toxicity of the eel extracts to developing *F. heteroclitus*. First, some classes of POPs, including DLCs, were lost in different proportions during eel extracts preparation (table 14). For example, some OCPs were almost totally destroyed by the acidic treatment and consequently their potential embryotoxic effects were not taken into account by the present study. There is a need to develop techniques to prepare representative organic extracts of eel tissues that include all classes of chemicals. Secondly, we have only examined short-term effects during a critical period of embryolarval development, from fertilization to the onset of feeding. Thus, the assay did not capture potential long-term or transgenerational effects, as demonstrated in sole (*Solea solea*) ELS exposed to PCB126 (Foekema et al., 2008) or in zebrafish ELS exposed to TCDD (Baker et al., 2013; Marit and Weber, 2012). Potential effects of POPs on cardiovascular or endocrine function, energetics, immune defense, larval metamorphosis, and sex differentiation (Johnson et al., 2013) were not examined in the present study. Thirdly, IVi injection of POPs may underestimates potential toxicity via maternal transfer since it does not measure effects associated with impaired transfer of nutrient and hormones associated with maternal exposure (Brooks et al., 1997).

Moreover, the relative sensitivity to DLCs of *A. rostrata* embryos compared to *F. heteroclitus* embryos is not known. Some studies suggest that eel species may be at least as sensitive to DLCs as rainbow trout (Cutler et al., 2011; Doering et al., 2012), one of the most TCDD-sensitive fish species (Elonen et al., 1998). Our previous findings on the marked variation in relative potencies of DLCs between *Fundulus* and trout species (Rigaud et al., 2013,

2014) indicate that there is a need to determine species-specific TEFs for various DLCs for risk assessment to *Anguilla* species. Our exploratory study with a model euryhaline species emphasizes the need to assess DLCs toxicity in *A. rostrata* ELS since it is a major toxic component of POPs accumulated by Atlantic eels, as suggested by previous studies in *A. anguilla* (Palstra et al., 2006). Our results provide some information on the type of responses that could be assessed in *Anguilla* leptocephali eventually captured in the Sargasso Sea, including EROD induction, craniofacial deformities and starvation (indicative of impaired prey capture ability). Further studies with model species are needed to document long-term and delayed effects of eel extracts in fish ELS and to further develop biomarkers amenable to the field.

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CONCLUSION GÉNÉRALE

L'objectif général de la présente thèse était d'explorer le rôle possible du transfert maternel des polluants organiques persistants (POP) dans le déclin du recrutement des juvéniles de l'anguille d'Amérique vers le lac Ontario. Pour cela, les variations temporelles de la toxicité de mélanges complexes de POP extraits d'anguilles femelles capturées dans le lac Ontario en 1988, 1998 et 2008 ont été mesurées chez les embryons de *Fundulus heteroclitus* par injection intravitelline (IVi). En guise de travaux préliminaires, des embryons de *F. heteroclitus* ont été exposés par injection IVi à des doses sous-létales de plusieurs POP historiquement mesurés dans les tissus des poissons du lac Ontario, y compris l'anguille, incluant certains composés apparentés aux dioxines (TCDD, BPC126, BPC77 et 2,3,4,7,8-PnCDF), deux BPC non-coplanaires (BPC52 et BPC110) ainsi qu'un mélange technique de BPC, l'Aroclor 1254. es trois hypothèses de recherche de la présente thèse sont énoncées en introduction, à la page 44.

Les résultats présentés dans les articles 1 et 2 permettent de rejeter la première hypothèse. À l'exception des deux BPC non-coplanaires testés (le BPC52 et le BPC110, qui n'ont eu aucun effet embryotoxique chez *F. heteroclitus* à des doses réalistes d'un point de vue environnemental), les autres composés testés, tous coplanaires (TCDD, 2,3,4,7,8-PnCDF, BPC126 et BPC77), ainsi que l'Aroclor 1254, ont tous présenté un patron de réponses embryotoxiques sous-létales similaire. Ces composés ont tous induit chez des larves de *F. heteroclitus* à 16 jours post-fécondation : un retard de croissance, des malformations crânio-faciales, une induction de l'activité EROD et une réduction de leur capacité de prédation, sans altération de leur activité locomotrice de base. L'altération de la capacité de prédation a été observée à des doses non nécessairement associées à une augmentation de la prévalence des malformations crânio-faciales, par exemple dès 1250 pg g^{-1} p.h. dans le cas de la TCDD et du BPC126. De plus, dans le cas du BPC126, les effets observés l'ont été à des doses réalistes d'un point de vue environnemental, du même ordre de grandeur que les concentrations me-

surées dans les tissus des touladis ou des anguilles du lac Ontario à la fin des années 1980 (Byer, 2013; Cook et al., 2003), c'est-à-dire comprises entre quelques centaines et quelques milliers de pg g^{-1} p.h.

De plus, les résultats présentés dans les articles 1 et 2 suggèrent que les FET de l'OMS pour le BPC126 et le 2,3,4,7,8-PnCDF chez les poissons, basés sur des études d'embryo-mortalité chez les salmonidés (Van den Berg et al., 1998), pourraient ne pas être applicables pour prédire le risque de toxicité chez d'autres espèces de poissons, à des doses sous-létales et réalistes d'un point de vue environnemental. Les potentiels toxiques relatifs de ces deux composés par rapport à la TCDD pour l'induction de l'activité EROD chez les jeunes stades de vie de *F. heteroclitus* étaient respectivement égaux à 0.71 et 2.40, soit environ 140 et 5 fois plus élevés que les FET de référence de l'OMS. Ce résultat souligne à quel point il est nécessaire de réactualiser ces FET chez les poissons, en générant des estimations de potentiels toxiques relatifs pour une plus large variété d'espèces de poissons, à des doses réalistes d'un point de vue environnemental.

Les résultats présentés dans le troisième article ne permettent pas de rejeter totalement la seconde hypothèse. En effet, les extraits organiques tissulaires d'anguilles du lac Ontario capturées en 1988, 1998 et 2008, bien que présentant un patron de réponses embryotoxiques sous-létales similaires aux composés coplanaires cités plus haut et à l'Aroclor 1254, n'avaient pas tous la même toxicité, cette dernière étant significativement plus faible dans le cas des extraits de 2008. Ces résultats sont conformes aux résultats de caractérisation chimique présentés par Byer (2013) et suggèrent que les composés apparentés aux dioxines présents dans les extraits, tout particulièrement le BPC126, sont les principaux contributeurs à l'embryotoxicité observée chez *F. heteroclitus*. L'activité EROD était la réponse la plus sensible, avec une induction significative dès 1 EEQ g^{-1} p.h. pour les extraits de 1988 et 1998, et dès 10 EEQ g^{-1} p.h. pour ceux de 2008. L'altération de la capacité de prédation n'a été significative qu'à partir de 5 et 10 EEQ g^{-1} p.h. pour les extraits de 1988 et 1998, respectivement. Ces concentrations sont théoriquement 5 à 10 fois supérieures à celles mesurées dans les tis-

sus des anguilles du lac Ontario. Néanmoins, un certain nombre de facteurs peuvent laisser penser que nos travaux surestiment ou sous-estiment la toxicité réelle des extraits d'anguilles (voir ci-dessous), et les seuils cités précédemment sont donc sujets à discussion.

La troisième hypothèse peut être rejetée à la lumière des résultats présentés dans le troisième article. Il a été impossible de prédire l'embryotoxicité observée avec les extraits d'anguilles en appliquant le concept d'EQT-TCDD. Les EQT-TCDD des extraits ont été calculés à partir de leurs concentrations pour certains composés apparentés aux dioxines (TCDD, BPC126 et 2,3,4,7,8-PnCDF) et de leurs potentiels toxiques relatifs chez *F. heteroclitus* (1, 0.71 et 2.40, respectivement). Ces EQT-TCDD basés sur les concentrations chimiques mesurées dans les extraits surestimaient systématiquement leur capacité à induire l'activité EROD chez les larves de *F. heteroclitus* au cours de notre étude. Ce phénomène a été également observé avec l'Aroclor 1254 au cours des travaux présentés dans le second article. Comme discuté dans les articles 2 et 3, il est possible qu'une compétition pour le récepteur Ah ou que des interactions entre les différents congénères de BPC ou les différentes classes de POP puissent atténuer la toxicité des composés apparentés aux dioxines présent dans un mélange, qu'il s'agisse d'un mélange technique tel que l'Aroclor ou d'un mélange plus complexe tel qu'un extrait tissulaire d'anguille.

Les travaux présentés au cours de cette étude sont les premiers à démontrer des effets embryotoxiques des POP accumulés dans les tissus des anguilles du lac Ontario. Nos résultats soutiennent l'hypothèse selon laquelle la contamination des anguilles du lac Ontario par les POP au cours des années 1980 et 1990, et tout particulièrement celle par les composés apparentés aux dioxines, aurait pu jouer un rôle dans la baisse de recrutement de l'espèce en altérant des paramètres pertinents d'un point de vue environnemental tels que la capacité de prédation ou la croissance des larves. Nos résultats soulèvent néanmoins plusieurs interrogations qui ont le mérite d'ouvrir des perspectives pour des études futures. Parmi ces interrogations, plusieurs facteurs peuvent limiter la portée de nos travaux en surestimant ou en sous-estimant le potentiel embryotoxique réel des mélanges organiques complexes accu-

mulés par les anguilles du lac Ontario :

1. De nombreux POP, incluant les composés apparentés aux dioxines, ont été perdus dans des proportions plus ou moins importantes au cours de la préparation des extraits. Cette perte de composés peut avoir altéré la toxicité des extraits dans un sens (diminution) ou dans l'autre (augmentation), selon la nature des interactions entre les composés.
2. Notre étude ne considère qu'un nombre limité d'anguilles capturées dans un nombre limité de sites, pour une période déterminée (1988-2008). Ces anguilles ne sont pas nécessairement représentatives de l'ensemble des anguilles du lac Ontario, et on peut supposer que les anguilles argentées ayant quitté le lac Ontario 10 ans plus tôt (1978) aient été plus contaminées puisqu'elles auraient grandi au moment du pic de contamination (Cook et al., 2003). Il est également possible que des anguilles capturées au sein d'autres sites du lac Ontario auraient été plus ou moins contaminées.
3. Notre étude se base sur des concentrations chimiques mesurées dans des homogénats d'anguilles entières n'ayant pas encore amorcé leur migration. Au cours de la migration qui précède la ponte, les anguilles mobilisent leurs réserves énergétiques en consommant leurs lipides, sans pour autant éliminer d'avantage de POP. De ce fait, les concentrations en POP dans les gonades et les œufs des anguilles peuvent être multipliées par un facteur 1.4 environ (Hodson et al., 1994). Il est donc possible que les concentrations en POP dans les œufs soient supérieures à celles mesurées dans les homogénats d'anguilles. Cela n'a jamais été vérifié puisque jamais des œufs d'anguilles n'ont été prélevés en mer.
4. Notre étude ne tient pas compte des effets à long terme (immunotoxicité, perturbations endocriniennes, etc.) et transgénérationnels possibles des POP (Foekema et al., 2008).
5. L'injection intravitelline ne mime pas tous les effets possibles d'un véritable transfert maternel de POP (distribution des POPs dans les tissus intra et extra-embryonnaires, altération de la qualité des œufs, du transfert de nutriments, etc.) (Brooks et al., 1997; Foekema et al., 2008).

6. La sensibilité relative des anguilles aux composés apparentés aux dioxines par rapport au choquemort n'est pas connue. À la lumière de certaines études, il est possible que les anguilles soient au moins aussi sensibles à la TCDD que les salmonidés (Cutler et al., 2011; Doering et al., 2012), qui figurent parmi les poissons les plus sensibles connus (Elonen et al., 1998).
7. Les effets combinés des POP et de déficiences nutritionnelles telles que celle en thiamine, documentée chez certains salmonidés des Grands Lacs, sont aussi à considérer (Brown et al., 2005; Couillard, 2009).

Pour répondre à ces différents points, plusieurs travaux futurs sont envisageables. Aussitôt que les jeunes stades de vie des anguilles (embryons et leptocéphales) seront aisément utilisables en laboratoire, il sera nécessaire de déterminer leur sensibilité aux composés apparentés aux dioxines ainsi que des FET spécifiques à l'espèce. En attendant que cela soit possible, les potentiels toxiques relatifs de certains composés apparentés aux dioxines (notamment le BPC126) pourraient être estimés chez des civelles ou anguilles jaunes par injection intrapéritonéale. Il serait également envisageable d'étudier les effets toxiques à long terme et transgénérationnels d'extraits organiques tissulaires d'anguilles chez des poissons modèles, tout en améliorant si possible les méthodes d'extraction de manière à perdre le moins de contaminants possible pendant la préparation des extraits.

Nos résultats fournissent également une gamme de biomarqueurs qui pourraient être utilisés sur le terrain, directement sur des leptocéphales capturés en mer des Sargasses, en mesurant par exemple l'induction de l'activité EROD, en recherchant d'éventuelles malformations crânio-faciales ou encore des signes de malnutrition, qui pourraient indiquer une éventuelle incapacité à capturer des proies. Une autre manière de procéder consisterait à capturer des anguilles argentées femelles à différents sites (témoins et contaminés), de les faire pondre artificiellement en captivité, comme cela est déjà possible (Oliveira et Hable, 2010), puis de comparer la survie embryo-larvaire, la capacité des leptocéphales à capturer des proies, etc., en relation avec la contamination des anguilles des différents sites par les

composés apparentés aux dioxines, qui serait mesurée par analyse chimique. Un tel projet est bien évidemment dépendant de notre capacité à maintenir des leptocéphales vivants en captivité.

Malgré sa raréfaction au cours des dernières décennies en Ontario et au Québec, c'est à dire à la limite septentrionale de l'aire de répartition de l'espèce, l'anguille d'Amérique reste relativement abondante ailleurs, tel qu'au centre de sa zone de distribution (DFO, 2010). L'anguille d'Amérique est une espèce panmictique ne présentant qu'une seule population, génétiquement identique (Côté et al., 2013). Dans ce contexte, il est difficile de comprendre pourquoi la raréfaction de l'espèce n'a été observée de manière si prononcée que dans le système Saint-Laurent et pas ailleurs. Néanmoins, malgré la panmixie, les anguilles d'Amérique argentées prêtes à entamer leur migration présentent des caractéristiques propres qui dépendent de leur zone géographique : par exemple, les anguilles femelles seront mâtures à une taille plus élevée à mesure que la distance à parcourir pour migrer vers la mer des Sargasses augmente (Jessop, 2010). L'encemencement dans le lac Ontario d'anguilles dont le site d'origine est plus proche de la mer des Sargasses que ne l'est le lac Ontario a pour effet de pertuber ce gradient écologique (Couillard et al., 2014) : les anguilles encemencées ne se développent pas suffisamment (plus petites, plus légères et moins de réserves lipidiques) comparativement aux anguilles sauvages du lac Ontario (Couillard et al., 2014), possiblement à cause de différentes pressions de sélection en début de vie. Il est possible que la mortalité sélective plus élevée des anguilles (et/ou de leurs leptocéphales) à forte propension migratoire dont font partie les anguilles du lac Ontario ait pu perturber l'équilibre évolutif stable de sorte que le génotype fortement migratoire s'est raréfié au sein de la population panmictique. Ceci pourrait expliquer le déclin plus prononcé au lac Ontario qu'ailleurs dans l'aire de répartition de l'espèce.

La portée de nos travaux ne se limite pas seulement à l'anguille d'Amérique. Bien que les concentrations des polluants organiques persistants aient également diminué au cours des dernières décennies dans les tissus des anguilles européennes (*Anguilla anguilla*) (de Boer

et al., 2010; Maes et al., 2008), des résultats récents suggèrent que les concentrations en composés apparentés aux dioxines sont encore élevées chez ces dernières. Par exemple, Byer (2013) a rapporté des concentrations en BPC126 égales à 304 ± 181 pg g^{-1} p.h. chez des anguilles capturées en 2009 en Belgique, des concentrations similaires à celles mesurées en 1988 et 1998 chez les anguilles du lac Ontario utilisées au cours de la présente étude (respectivement 492 ± 102 et 370 ± 75 pg g^{-1} p.h. dans les homogénats, voir tableau 14). À notre connaissance, dans le cas de l'anguille japonaise (*Anguilla japonica*), aucune étude récente n'a rapporté les concentrations en dioxines et composés apparentés. Des projets de travaux tels que ceux cités précédemment dans le cas de l'anguille d'Amérique sont également requis chez les espèces d'Europe et d'Asie pour évaluer les menaces passées, présentes et futures qui ont pesé ou pèsent encore sur elles.

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