UNIVERSITÉ DU QUÉBEC À MONTRÉAL

ÉVALUATION ET DÉTECTION DES EFFETS DE MODULATEURS ENDOCRINIENS JUVÉNOÏDES CHEZ *DAPHNIA MAGNA* TESTÉS AVEC LES EFFLUENTS D'USINE DE PÂTE ET PAPIER

MÉMOIRE

PRÉSENTÉ

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AVANT-PROPOS

Ce mémoire est divisé en deux articles scientifiques. Le premier a pour titre : «Testing for juvenoid endocrine disruptors in *Daphnia magna* : neonate phenotype and Hb2 gene induction». Il est en préparation pour être soumis à la revue *Aquatic Toxicology*. Le deuxième article a pour titre : « Potential juvenile and ecdysteroid disrupting effects of pulp mill effluents and juvabione in *Daphnia magna*». Il ne sera pas soumis dans sa forme actuelle, mais peut-être après modifications. Pour le premier article, les auteurs sont Marie-Ève Roy et Philip Spear puisqu'ils ont tous les deux participés à la rédaction. Pour le deuxième article, les auteurs sont Marie-Ève Roy, Philip Spear et Tibor Kovacs. Tibor Kovacs est un auteur associé, en raison de son aide et de ses expertises dans le domaine. Je suis la première auteure de ces deux articles. J'ai aussi pensé et réalisé les manipulations, les analyses et les expériences.

Avant d'entreprendre les manipulations pour les expériences, j'ai premièrement travaillé pendant trois mois à l'entretien et la culture de *D.magna* et *Ceriodaphnia dubia* à PAPRICAN (Pulp And Paper Reseach Institude of Canada). Ensuite, j'ai fait des tests préparatoires dans les laboratoires de l'UQAM. Finalement, il me fut possible de réaliser les expériences prévues aux deux endroits.

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LISTE DES ABRÉVIATIONS, SIGLES ET ACRONYMES

ADN	Acide désoxyribonucléique
ARN	Acide ribonucléique
ARNm	ARN messager
cDNA	*Complementary deoxyribonucleic acid* (ADN complémentaire)
20-E	20-ecdysone
EcR	*Ecdysteroid receptor* (récepteur d'ecdystéroïde)
EcRRE	*Ecdysteroid receptor response element* (élément de réponse du récepteur d'ecdystéroïde)
EDC	*Endocrine disrupting chemicals* (modulateur du système endocrinien)
EPO	*Erythropoietin* (érythropoietin)
HIF-1	*Hypoxia inducible factor 1* (facteur de l'hypoxie-1)
HRE	*Hypoxia response element* (élément de réponse de l'hypoxie)
JH	*Juvenile hormone* (hormone juvénile)
JHR	*Juvenile hormone receptor* (récepteur de l'hormone juvénile)
JRE	*Juvenile hormone response element* (élément de réponse de l'hormone juvénile)
Mf	*Methyl farnesoate* (méthyle farnésoate)
MIH	*Moult-inhibiting hormone* (hormone inhibitrice de la mue)
MOIH	*Mandibular organ-inhibiting hormone* (hormone inhibitrice de l'organe mandibulaire)
mRNA	*Messenger ribonucleic acid* (voir ARNm)
PCR	*Polymerase chain reaction* (amplification en chaîne par polymérase)
RNA	*Ribonucleic acid* (voir ARN)

Multiplicateurs

d	$X\ 10^{-1}$
m	$X\ 10^{-3}$
μ	$X\ 10^{-6}$
n	$X\ 10^{-9}$

LISTE DES SYMBOLES

%	Pourcentage
260 nm	L'onde de 260 nm
IC25	Concentration causant une inhibition de 25% (µg/L)
LC50	Concentration produisant 50% de mortalité (µg/L)
v/v	Volume / volume

Unités	**Nom complet**	**Mesure**	**Équivalence**
bp	*base pair* (paire de base)	Longueur d'un enchaînement polynucléotidique en acides nucléiques monocaténaires	
°C	degrés Celsius	Température	
Da	dalton	Masse atomique	1 Da = 1.650 X 10 -24 g
G	Constante d'accélération gravitationnelle	Vitesse	G = 9.8 m/s
g	gramme	Masse	
h	heure	Temps	
L	litre	Volume	1 L = 0.001 mètre cube
lux	lumens	Intensité lumineuse	
M	molaire	Concentration	1 M = 1 mole / litre
m	mètre	Longueur	
min	minute	Temps	
mhos	siemens	Conductivité	1/ohm

RNase	Ribonucléase
RT	*Reverse transcriptase* (transcriptase inverse)
RXR	*Retinoic receptor X* (récepteur rétinoïque X)
TMP	*Thermomechanical pulp* (pâte thermomécanique)
USP	*Ultraspiracle receptor* (récepteur ultra spiracle)

Organismes

FQRNT	Fonds Québécois de la Recherche sur la Nature et les Technologies
OECD	Organisation de Coopération et de Développement Économiques
PAPRICAN	*Pulp and Paper Research Institute of Canada*
SETAC	*Society of Environmental Toxicology and Chemistry*

Gènes

Hb2	*Haemoglobin gene 2* (gène pour l'hémoglobine 2)

Autres abréviations

BOD	*Biochemical oxygen demand* (Demande biochimique en oxygène)
DO	*Dissolved oxygen* (Oxygène dissout)
RFA	*Resin and fatty acid* (Acide gras et résine)
pH	Potentiel d'hydrogène
UV	Ultraviolet

RÉSUMÉ

Les perturbateurs endocriniens ont la capacité d'altérer les systèmes hormonaux des invertébrés. Lorsqu'elles sont rejetées dans le milieu aquatique, elles peuvent moduler le signalement de systèmes hormonaux centraux et, par conséquent, avoir un impact sur la physiologie et l'aptitude phénotypique des invertébrés aquatiques. Le changement de sexe et l'accumulation de l'hémoglobine chez *Daphnia magna* sont connus pour être des réponses aux perturbateurs endocriniens affectant le système de l'hormone juvénile. Cependant, dans plusieurs cas, notamment avec les effluents d'usines de pâte et papier et avec les substances pures des arbres, la détection, les effets et les mécanismes d'action de ces pollutions n'ont jamais été évalués. Notre premier objectif est alors de développer et de tester un bioindicateur de pollutions endocriniennes juvéniles chez le crustacé *D. magna*. Dans ce but, la modification de l'expression de l'hémoglobine impliquant le récepteur de l'hormone juvénile (JHR) est évaluée en utilisant la méthode PCR avec des amorces spécifiques au gène Hb2 chez les daphnies. De plus, la mesure de l'augmentation du phénotype mâle lorsque les daphnies sont exposées à des effluents d'usine de pâte et papier ou à une substance pure des arbres est faite par la méthode comparative. Nos résultats montrent que le pyriproxyfène, qui est un analogue de l'hormone juvénile servant de contrôle positif, induit plus de 2 fois le gène Hb2 à 1.6 µg/L et cause aussi une augmentation du phénotype mâle chez les rejetons. Les effluents ayant subi un traitement primaire (10% v/v), induisent plus de 2 fois l'expression du gène Hb2, contrairement aux effluents avec traitement secondaire (50% v/v) qui ne cause pas d'induction. Un analogue de l'hormone juvénile détecté dans les effluents primaires, la juvabione, est capable d'augmenter l'expression du gène Hb2, à de faibles concentrations (3.75 µg/L). Une exposition prolongée causant une diminution du niveau d'oxygène cause cependant une faible augmentation de l'expression du gène Hb2. Nos résultats indiquent que l'induction du gène Hb2 peut être utilisée afin de détecter les analogues de l'hormone juvénile; les juvénoïdes. Le second objectif est de détecter les effets physiologiques et phénotypiques des pollutions potentiellement juvénoïdes. Afin de déterminer ces effets sur la première génération de *D. magna,* nous avons mesuré le temps de mue des femelles et le nombre de néonates par portée. Pour leurs progénitures, le changement de phénotype sexuel sous le contrôle de l'hormone juvénile, la taille et la mortalité sont les paramètres testés. Avec les effluents primaires (2.5% v/v) un nouveau phénotype a été repéré : le phénotype "juvénile". De plus, une prolongation de la mue et une diminution du nombre de néonates par femelle furent observées. En revanche, avec les effluents secondaires, trois des quatres effluents testés n'ont pas démontré ces effets. Nos résultats suggèrent que ces différences entre les deux types d'effluents peuvent être attribuées au fait que le traitement secondaire diminue fortement la concentration de certaines substances naturelles des arbres comme la juvabione. En effet, la juvabione, à faible concentration (2.25 µg/L), empêche le développement de néonates et augmente le temps de la mue. L'analyse des résultats démontre aussi l'importance de faire des tests sur les adultes et sur les néonates pour de meilleurs résultats. En conclusion, l'induction du gène Hb2 combinée à des indicateurs biologiques comme le temps de la mue et le changement de phénotype sexuel, ont permis de détecter les effets potentiellement juvénoïdes et anti-ecdystéroïdes des effluents primaires et de la

juvabione.

Mots clés: Hormone juvénile, Gène Hb2, Phénotype, Effluents d'usines de pâte et papier, *Daphnia magna*

INTRODUCTION GÉNÉRALE

Il fut un temps, où l'on croyait que chaque récepteur était activé par une seule hormone (Gustafsson, 1999). De nos jours, nous savons que plusieurs ligands endogènes peuvent lier le même récepteur et que la communication existe entre récepteurs ('cross-talk'). De plus, des modulateurs endocriniens environnementaux ont la capacité de lier des récepteurs et d'activer la transcription génétique (Hester et Harrison, 1999). En contaminant les milieux aquatiques ou en s'accumulant dans la chaîne alimentaire, ces modulateurs peuvent avoir des conséquences au niveau des populations d'organismes s'y trouvant (Araki *et al.*, 2000; Bowerman *et al.*, 2000; Environnement Canada, 2002; Guillette *et al.*, 1994). Ces modulateurs hormonaux sont connus pour affecter le système endocrinien des invertébrés (Hester et Harrison, 1999; LeBlanc et *al.*, 1999; Nice, 2005; Zou et Fingerman, 1997). Cependant, plusieurs substances ayant la possibilité d'être des modulateurs endocriniens chez les invertébrés, comme les effluents rejetés en milieux aquatiques, n'ont pas de mécanisme ciblé afin d'expliquer leurs effets (Environnement Canada, 2002; Taylor *et al.*, 1996; Walker *et al.*, 2005). De plus, les effets endocriniens sont variés et parfois difficilement détectables chez ces organismes (Defur, 2004; Zou et McKeown, 2005). Il est donc difficile de connaître les répercussions des modulateurs endocriniens sur les invertébrés et sur leurs populations.

1. Le système hormonal des arthropodes

Parmi les invertébrés, les arthropodes sont le groupe le plus abondant et le plus diversifié avec plus d'un million d'espèces recensées (Tobe et Bendena, 1999). Ce groupe inclut les insectes et les crustacés. Le système hormonal des arthropodes est régulé par des facteurs externes (ex. l'environnement) qui peuvent être intégrés par l'intermédiaire du système nerveux central. Il s'en suit un relâchement de neuropeptides et la production d'hormones par les glandes endocrines (Cuzin-Roudy et Saleuddin, 1989). Chez les crustacés, deux des principales glandes endocrines sont l'organe-y et l'organe mandibulaire. L'organe-y sert à la

biosynthèse des ecdystéroïdes et sécrète l'ecdysone. Pour sa part, l'organe mandibulaire sécrète l'hormone juvénile (Quackenbush, 1986). Ces deux types d'hormone contrôlent plusieurs aspects du développement (ex. mue, métamorphose, embryogenèse) et de la reproduction chez les arthropodes (Chang *et al.*, 2001; Ghbeish *et al.*, 2001; McCourt *et al.*, 2005). Vu le rôle central des hormones juvéniles et des ecdystéroides (LeBlanc, 2007), la section suivante portera principalement sur ces deux hormones, afin de mieux comprendre leurs modes d'actions.

1.1. Le signalement des hormones juvéniles et des ecdystéroïdes

Les arthropodes existent depuis plus de 500 millions d'années et l'on suppose que les hormones juvéniles furent premièrement utilisées comme régulateur de la reproduction (Tobe et Bendena, 1999). Il est possible d'observer le caractère évolutif similaire des hormones juvéniles entre les insectes et les crustacés. Par exemple, l'organe des insectes produisant les hormones juvéniles (le corpus allata) et celui des crustacés (organe mandibulaire) ont la même origine embryonnaire (Tobe et Bendena, 1999). La forme non oxydée de l'hormone juvénile 3 des insectes; soit le méthyle farnésoate (Mf), est l'hormone juvénile des crustacés (LeBlanc, 2007). Le Mf a été mesuré chez plus de 30 espèces de crustacés, dont les crabes, les crevettes et la daphnie (Tobe et Bendena, 1999).

Malgré plusieurs recherches, l'identification d'un récepteur pour l'hormone juvénile chez les insectes et pour son homologue chez les crustacés reste à confirmer (Wozniack *et al.*, 2004). Chez plusieurs insectes comme la drosophile, un candidat pour l'hormone juvénile 3 serait le récepteur rétinoïque X (RXR), originellement appelé ultraspiracle (USP) * (Fang *et al.*, 2005;

* Le liens entre le USP et le RXR n'est pas le même selon les études, certaines appellent USP l'homologue du récepteur RXR ou utilise simplement le terme USP (Fang *et al.*, 2005; Jones et Sharp 1997; Jones *et al.*, 2001; Barchuk *et al.*, 2004; Wu *et al.*, 2004; Antoniewski *et al.*, 1996). Cependant, en accord avec les études plus récentes (LeBlanc, 2007), le terme RXR sera employé pour désigner à la fois le récepteur USP et RXR dans ce texte.

Jones et Sharp, 1997; Jones *et al.*, 2001). Il est possible que le récepteur du méthyle farnésoate aussi appelé récepteur d'hormone juvénile (JHR), soit en fait le RXR (LeBlanc, 2007). Cependant, le mécanisme d'action du RXR n'a pas été démontré ni chez les insectes (Wozniak *et al.*, 2004), ni chez les crustacés (LeBlanc, 2007). Par contre, un élément de réponse du récepteur de l'hormone juvénile (JRE) a été détecté. La présence de celui-ci chez les insectes et les crustacés indique la possibilité d'un mécanisme d'action commun pour le signalement de l'hormone juvénile (Gorr *et al.*, 2006).

Une des plus importantes formes d'ecdystéroïdes est le 20-hydroxyecdysone (20-E) (Cuzin-Roudy et Saleuddin, 1989; Dauphin-Villemant *et al.*, 1997). Chez les crustacés, les ecdystéroïdes comme le 20-E sont sous le contrôle négatif de l'hormone inhibitrice de la mue (MIH) (LeBlanc, 2007), qui maintient un niveau bas d'ecdystéroïdes entre les mues. Le récepteur d'ecdystéroïde (EcR), désigné par le gène NR1h1 est activé par son propre ligand (ex. 20-E) et par la formation d'un hétérodimère avec le récepteur RXR sans ligand (Barchuk *et al.*, 2004; Wu *et al.*, 2004). Ce complexe se lie à des éléments de réponse du récepteur de l'ecdystéroïde (EcRE) (Antoniewski *et al.*, 1996) de plusieurs gènes afin de réguler leurs activités. Des gènes comme HR3, HR38, E75 sont régularisés selon ce modèle (Hiruma et Riddiford, 2004; Toya *et al.*, 2002). Le signalement normal du EcR implique donc que le RXR ne soit pas lié à ses ligands (LeBlanc, 2007).

Les hormones juvéniles chez des arthropodes sont régularisées de façon cyclique avec les ecdystéroïdes. Chez les insectes et les crustacés, quand la quantité d'hormones juvéniles change brusquement, le cycle normal créé par les ecdystéroïdes (ex. la mue) est altéré (Quackenbush, 1986). L'hormone juvénile les crustacés (Mf) est impliquée dans le contrôle de la sécrétion de l'organe–y et donc, dans la synthèse des ecdystéroïdes. Les hormones juvéniles ont aussi des activités anti-ecdysteroïdales, cependant le mécanisme d'action n'a pas été déterminé (Mu et LeBlanc, 2004). Il existe peu de méthodes de dépistage des modulateurs de l'hormone juvénile et des ecdystéroïdes, malgré le besoin grandissant de les détecter, notamment chez les crustacés (Tatarazani et Oda, 2007; Zou et McKeown, 2005).

2. Indicateurs biologiques et génétiques de l'hormone juvénile chez les crustacés

2.1 Daphnia magna comme modèle chez les crustacés

La daphnie est un crustacé aquatique qui se nourrit de plancton; l'absorption de substances chimiques se fait à travers la membrane épithéliale. La daphnie agit de façon similaire en laboratoire ou en nature et se retrouve dans presque tous les cours d'eau permanents (Tatarazako et Oda, 2007). Elle occupe aussi une place importante dans la chaîne alimentaire puisqu'elle fait partie du festin des poissons et des prédateurs invertébrés. C'est un organisme utilisé pour la réglementation de la toxicité des effluents d'usines (Weber, 1993). Les mécanismes physiologiques dont le changement de sexe, ainsi que le mécanisme d'induction du gène de l'hémoglobine, décrit ci-dessous chez la daphnie, sont connu pour être des réponses aux actions de l'hormone juvénile.

2.2. Induction de l'hémoglobine chez la daphnie

Pour *D. magna*, le récepteur JHR non identifié serait un récepteur nucléaire monomérique de 52 kDa qui se lie à un élément de réponse de l'hormone juvénile (JRE) occupant la position *cis* (Gorr *et al.*, 2004). Pour sa part, le JRE a été identifié et consiste en un élément de réponse centrale stéroïde/rétinoïde et en une région riche en 5` AT. Chez la daphnie, le JRE a été repéré sur un gène pour l'augmentation de l'hémoglobine : le Hb2 (Gorr *et al.*, 2006). Gorr *et al.* (2006) ont démontré que ce gène peut être activé par le complexe de liaison entre une substance juvénile, son récepteur (JHR) et son élément de réponse (JRE). Les seuls ligands actuellement testés pour ce mécanisme sont le méthyle farnésoate et le pyriproxyfène; le pyriproxyfène étant le plus efficace pour l'induction du gène Hb2 (Gorr *et al.*, 2006). Le promoteur du gène Hb2 chez la daphnie contient un JRE où le récepteur d'hormones juvéniles (JHR) peut se lier, mais aussi un élément de réponse pour l'hypoxie (HRE) où le facteur de transduction pour l'hypoxie (HIF) peut se lier.

L'HRE et le JRE sont séparés par 10 pb et forment un système de réponse alternatif. Lorsque le niveau d'oxygène est normal, le gène de Hb2 est sous l'influence de JRE. Dans le cas où le niveau d'oxygène est bas, le gène Hb2 est alors sous l'influence de HRE, afin de permettre une augmentation d'hémoglobine (Gorr *et al.*, 2004). Pour avoir cette augmentation, il faut que le HIF-1β et son dimère le HIF-1α mieux connu sous le nom de translocateur du récepteur aux hydrocarbures arylées (ARNT) soient liés à un HRE. Contrairement au HIF-1α, le HIF-1β impliqué dans les réponses oxydatives aux xénobiotiques, est constitutivement lié au HRE. Plusieurs mécanismes ont été étudiés afin d'expliquer l'action du HIF-1 α, mais ceux de l'hydroxylation de la proline sont les mieux acceptés. Ce mécanisme repose sur le fait qu'en présence d'oxygène, l'hydroxylation de la proline s'effectue, inactivant ainsi le HIF-1 α par dégradation. Lorsque le niveau d'oxygène est plus bas, la dégradation n'a pas lieu.

2.3 Effets physiologiques de l'hormone juvénile et production du phénotype mâle chez Daphnia magna

Chez les crustacés, l'hormone juvénile (Mf) est impliquée dans la maturation sexuelle, le comportement sexuel, la métamorphose et la morphogenèse des larves (Fingerman, 1997; LeBlanc *et al.*, 1999; Olmstead et LeBlanc, 2002; LeBlanc, 2007). Cependant, son effet sur le développement larvaire et la métamorphose n'est pas facilement prévisible puisque différentes études montrent des résultats opposés. Il stimule le développement larvaire dans certaines classes de crustacés et l'inhibe dans d'autres. Indirectement, le Mf peut aussi être impliqué dans le contrôle de la mue fait par les ecdystéroïdes appelé l'ecdysis (Chang *et al.*, 2001; Mu et LeBlanc, 2004).

Parmi les effets physiologiques du Mf chez la daphnie, de récentes études montrent que la production de mâles peut être un indicateur d'analogues de l'hormone juvénile (Tatarazako et Oda, 2007). Dans un environnement sain, les daphnies se reproduisent sans fécondation des œufs qui se développent en phénotype femelle à l'intérieur de quelques jours. Dans un environnement stressant (c.-à-d. diminution de la photopériode, agglomération et manque de

nourriture), il peut y avoir production de mâles (Baer et Owens, 1999) par l'intermédiaire du Mf. Ainsi, pour *Daphnia magna*, le Mf est le déterminant sexuel. En effet, l'oocyte exposé à cette hormone pendant la maturation ovarienne développe le phénotype mâle (Olmstead et LeBlanc, 2002).

La différence entre mâles et femelles est que les mâles sont plus petits, ils ont une morphologie différente pour les antennes et leur premier membre a un "crochet" (Hobaek et Larsson, 1990). Après seulement 24h, les mâles peuvent être distingués des femelles par la longueur et la forme de la première antenne (Tatarazako et Oda, 2007). Chez les branchiopodes qui incluent les daphnies, des observations antérieures révèlent que les portées sont constituées de mâles seulement ou de femelles seulement (Barker et Hebert, 1990). Cependant, une administration d'un niveau intermédiaire de Mf stimule la production de portées mixtes (Olmstead et LeBlanc, 2002) et même des individus appelés intersexes (LeBlanc, 2007). Lorsque les mâles fertilisent les femelles, il y a production de coquilles protectrices appelées épphipia. Ces coquilles sont conçues pour survivre à des conditions extrêmes pour une daphnie comme le gel et la sécheresse.

2.4 Liens entre le phénotype mâle et l'induction du gène Hb2 chez D. magna

Des corrélations directes ont été faites entre l'incidence du phénotype mâle et la régulation d'hémoglobine chez la daphnie (Rider *et al.*, 2005). La présence de phénotypes mâles est toujours corrélée avec l'augmentation d'hémoglobine, mais il est possible d'observer l'augmentation d'hémoglobine sans changement de phénotype male. Aussi, une substance (ex. bisphénol A) qui peut augmenter la dégradation de la sous-unité α de HIF-1, peut aussi augmenter la capacité du Mf à stimuler la production de mâles (Wang *et al.*, 2005). Cependant, les mécanismes qui permettraient d'expliquer ces liens ne sont pas encore bien déterminés (Gorr *et al.* 2006).

3. Les analogues de l'hormone juvénile

Les juvénoïdes sont des substances qui imitent les hormones juvéniles chez les insectes (Bede, 2000) et sont une catégorie de modulateurs endocriniens. Il est possible que les juvénoïdes aient des effets semblables chez les invertébrés aquatiques notamment les crustacés. Bien que certains effluents d'usines de pâte et papier et certains produits botaniques pourraient avoir des caractéristiques de substances juvénoïdes, les études réalisées sur la perturbation du signalement des hormones juvéniles chez les crustacés ont testé principalement des pesticides et des hormones juvéniles (LeBlanc *et al.*, 1999; Wang *et al.*, 2005).

3.1 Les pesticides

Certains insecticides sont synthétisés dans le but d'interagir ou d'imiter les effets des hormones juvéniles afin de contrôler les insectes nuisibles en agriculture et en foresterie (Wang *et al.*, 2005). Ceux appelés régulateur de croissance agissent sur plusieurs aspects du développement des insectes dont la métamorphose (Bortolotti *et al.*,2005). Le méthoprène synthétique, certain de ses métabolites et le fénoxycarbe sont aussi connus comme étant capables d'avoir des effets sur le développement des insectes et des crustacés (Wang *et al.*, 2005, Horst et Walker, 1999). Pour le crustacé modèle *D. magna*, de faibles concentrations de pyriproxyfène, de fénoxycarbe, de méthoprène et d'hydroprène stimulent aussi la production des mâles (Tatarazaki et Oda, 2007; Wang *et al.*, 2005). Par ailleurs, d'autres pesticides utilisés en agriculture comme le fongicide fénarimol, sont plutôt connus pour leurs activités d'anti-ecdystéroïde. Chez les insectes et les crustacés, l'exposition d'embryons ou de larves à des substances affectant le système des ecdystéroïdes peut entraîner des déformations, des anormalités et la mort (Belai et Fekete, 2003; McKenney, 2005; Mu et LeBlanc, 2002; Tuberty et McKenney, 2005).

3.2 Les effluents d'usines de pâte et papier et leurs effets sur les crustacés

Les effluents d'usines de pâte et papier sont connus pour avoir des effets sur les organismes aquatiques (Environment Canada, 2002; Johnsen *et al.*, 2000; Kovacs *et al.*, 2005; Martel *et al.*, 2000; Martel *et al.*, 2002; Munkittrick et van der Kraak, 1994; Munkittrick *et al.*, 1997; Rissanen *et al.*, 1997). Pour citer quelques exemples avec les invertébrés, une étude sur la rivière St-François (Québec, Canada) réalisée entre 1998 et 2000 sur les moules d'eau douce démontre qu'il existe une diminution de taille ainsi qu'un problème de féminisation en aval des usines (Martel *et al.*, 2003). D'autres informations sur les moules d'eau douce adultes, *Elliptio buckleyi* et *Elliptio icterina*, exposée aux effluents d'usines de pâte et papier dans un système de traitement à débit continu, suggèrent qu'il y a un effet endocrinien significatif sur leur système reproducteur dû à cette exposition (Kernaghan *et al.*, 2001). Des déformations lors du développement larvaire d'insectes aquatiques ont aussi été observées lors d'exposition aux effluents d'usines de pâte et papier (Jacki *et al.*, SETAC Conference 2001). Toutefois, plusieurs tests utilisant les invertébrés pour la réglementation de la toxicité des effluents ne détectent pas certains modulateurs endocriniens (Environment Canada, 2002; Walker *et al.*, 2005).

Le procédé de mise en pâte thermomécanique (PTM) consiste à utiliser de la vapeur et de l'action mécanique afin de séparer les copeaux de bois en fibres individuelles (Environnement Québec 2002). Cette méthode est considérée comme non-chimique (Pokhrel et Viraraghavan, 2004). L'industrie papetière rejette un grand volume d'effluents dilués dans les cours d'eau. Au Québec pour l'année 2001 par exemple, le chiffre s'élève à environ 593 millions de mètres cubes (Environment Québec, 2002). La concentration des effluents d'usines de pâte et papier dans les eaux canadiennes peut varier entre 0.01 et 10% (Martel *et al.*, 2000). Le système de traitement des eaux est donc très important. Ainsi, la plupart des usines de pâte et papier thermomécaniques sont dotées d'un traitement secondaire consistant à utiliser des micro-organismes afin d'accélérer la décomposition naturelle des résidus organiques soit par activation de résidus organiques ou par aération stabilisation (Environnement Québec, 2002). Ce traitement permet de réduire une bonne proportion de

substances secondaires des arbres (Martel *et al.*, 1997), comme les acides résiniques et les composés phénoliques.

Avec les usines thermomécaniques, un nouvel intérêt est porté sur les effets des substances naturelles des arbres qui se retrouvent dans les effluents (Martel *et al.*, 2000). Parmi les principales essences d'arbres utilisées par les usines thermomécaniques aux Québec, on retrouve le sapin baumier (*Abies balsamea*) et l'épinette noire (*Picea mariana*) (Martel *et al.*, 1997). On retrouve aussi l'épinette blanche (*Picea glauca*), le pin blanc (*Pinus strobus*), le pin gris (*Pinus banksiana)*, le pin rouge (*Pinus resinosa*), la pruche *(Tsuja occidentalis*) et le peuplier faux-tremble (*Populus tremuloides)* (Hanneman *et al.*, 2002; O'Connor *et al.*, 1992). Les acides résiniques, les stérols, les stilbènes, les juvabiones, les polyphénols et les acides gras sont des substances provenant des arbres que l'on peut extraire des effluents (O'Connor *et al.*, 1992 ; Pranovich *et al.*, 2005; Verenich *et al.*, 2004; Willfor *et al.*, 2004 a). Parmi celles-ci, certaines sont des juvénoïdes comme la déhydrojuvabione, la juvabione, et l'épijuvabione (Bede, 2000). Les sapins baumiers (*Abies balsamea*) ont une concentration beaucoup plus élevée de juvabione que d'autres espèces d'arbres utilisées en industrie, malgré une bonne variation entre les sapins (Bowers *et al.*, 1966; Pichette *et al.*, 1998; S. Willfor *et al.*, 2004b).

3.3 Les substances naturelles des arbres et leurs effets chez les insectes et les crustacés

Les arbres ont développé de multiples façons de se défendre contre les insectes (Bede, 2000), utilisant des substances secondaires comme les terpénoïdes, dont certains possèdent de fortes ressemblances structurelles avec les hormones juvéniles des insectes. Les terpénoïdes, des dérivés biosynthétiques de l'isoprène (C5H8), sont le constituant majeur des huiles essentielles de plusieurs conifères. Parmi les terpénoïdes servant à la défense d'arbres, on retrouve la juvabione, son précurseur E-alpha-bisabolène, ainsi que E-beta-farnésène, E,E-farnésol et E,E-alpha-farnésène, qui peuvent être induit par une attaque d'insectes (Kelling et Bohlmann, 2006; Romeo, 2005). Par exemple, pour certains arbres, les concentrations de

substances comme la juvabione et la déhydrojuvabione augmentent lors d'attaques de pucerons (Fowler *et al.*, 2001). Ces substances peuvent ensuite servir à la protection des arbres.

Dans les années 1970, des études avaient déterminé que les substances avec des activités d'hormones juvéniles (ex. juvabione et l'hormone juvénile 3) peuvent bloquer le développement embryonnaire d'insectes (Zaoral et Slama, 1970) et empêcher l'éclosion des œufs chez la tordeuse d'épinette (*Choristoneura fumiferana*) (Retnakaran, 1970). Il est maintenant connu que l'application de terpénoïdes avec des activités juvénoïdes sur des œufs d'insectes peut causer des mâles stériles ou des larves qui ne deviennent pas adultes (Bede, 2000). C'est le cas de la juvabione et de la déhydrojuvabione, qui empêchent la formation d'adulte chez les Pyrrhocoridés (Whittaker et Feeny, 1971), et l'éclosion de presque tous les œufs chez d'autres insectes (Bede, 2000).

Le terme juvénoïde s'applique particulièrement aux insectes. Ainsi, peu d'informations sont disponibles sur les effets des juvénoïdes sur les invertébrés aquatiques notamment les crustacés. Cependant, une étude a décelé que de faible concentration de déhydrojuvabione (0.5 µg/L) et de juvabione (7 µg/L) sont capables d'avoir des effets chroniques sur la reproduction du crustacé *Ceriodaphnia dubia* (O'Connor *et al.*, 1992).

4. Objectifs de l'étude

Le premier objectif est de tester un bioindicateur pour la perturbation endocrinienne des juvénoïdes en utilisant l'induction du gène Hb2 couplée avec le changement de phénotype sexuel chez le crustacé *D. magna*. La mesure de l'expression d'un gène de l'hémoglobine Hb2, permet de voir si les effets juvénoïdes sont produits en se liant au récepteur de l'hormone juvénile activé par le complexe juvénoïde/JHR. Cet objectif permet en même temps de déterminer si les effluents d'usines de pâte et papier et les substances naturelles des arbres (ex. juvabione) agissent comme des analogues de l'hormone juvénile chez les

crustacés, notamment la daphnie.

Le second objectif découle du premier et consiste à détecter les effets physiologiques des perturbateurs juvénoïdes. Il répond directement à la question : quel est l'impact des effluents d'usines de pâte et papier sur les daphnies adultes et sur leurs progénitures. Cet objectif a aussi pour but de préciser l'influence des divers traitements d'effluents et de la juvabione sur ces effets physiologiques. Il permet aussi d'évaluer l'utilité des indicateurs biologiques pour le développement de bioessais avec les invertébrés aquatiques.

ARTICLE I

Testing for juvenoid endocrine disruptors in *Daphnia magna* : neonate phenotype and Hb2 gene induction

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Abstract : Among the responses of *Daphnia* (*D. magna*) to environmental stressors are changes in sexual phenotype and haemoglobin accumulation. The underlying mechanisms, though only partially understood, apparently involve the juvenile hormone signalling mechanism which is linked to genetic regulation of haemoglobin synthesis. Sources of juvenile hormone analogs or juvenoids in the environment include natural plant compounds that may be discharged by pulp and paper mill effluents as well as some pesticides. Experiments were undertaken to determine if the environmental juvenoids are capable of binding to the juvenile hormone receptor (JHR) and thereby cause sexual phenotype changes in *Daphnia* (i.e. more males) and a commensurate increase in haemoglobin Hb2 gene transcription. The pesticide pyriproxyfen at a concentration of 1.6 µg/L elicited more than a 2-fold induction of the gene Hb2 in gravid *Daphnia* and all neonates demonstrated the male phenotype. Exposure of adult *Daphnia* to 10% (v/v) primary treated pulp effluent from a thermomechanical pulp (TMP) mill, containing a variety of wood extracts including juvabiones, resulted in more than a 2-fold Hb2 induction. Adult *Daphnia* exposed to a 2.5% (v/v) concentration of this effluent produced neonates that were small in size and remained sexually immature. Juvabione, a component of the primary-treated TMP effluent, stopped *Daphnia* reproduction altogether at a concentration of 2.25 µg/L and induced the Hb2 gene at a concentration of 2.4 µg/L. Secondary treatment of the TMP effluent, which reduced juvabione to non-detectable levels, eliminated the effect on Hb2 even at a dilution of 50% (v/v). Nonetheless, secondary-treated effluents elicited varied developmental effects. The neonates produced by adult *Daphnia* exposed to one secondary-treated effluent out of four, resulted in 100% "juvenile" phenotype. Longer exposure to secondary treated effluents with noticeable decrease in oxygen concentration did slightly induce the Hb2 gene, possibly by the hypoxic pathway. Results of this study show that induction of the Hb2 gene combined with developmental endpoints such as sexual phenotype could be useful monitoring tools for arthropods exposed to juvenoid-like compounds.

Keywords : pyriproxyfen, sex ratio, haemoglobin, Hb2 gene, pulp and paper effluent

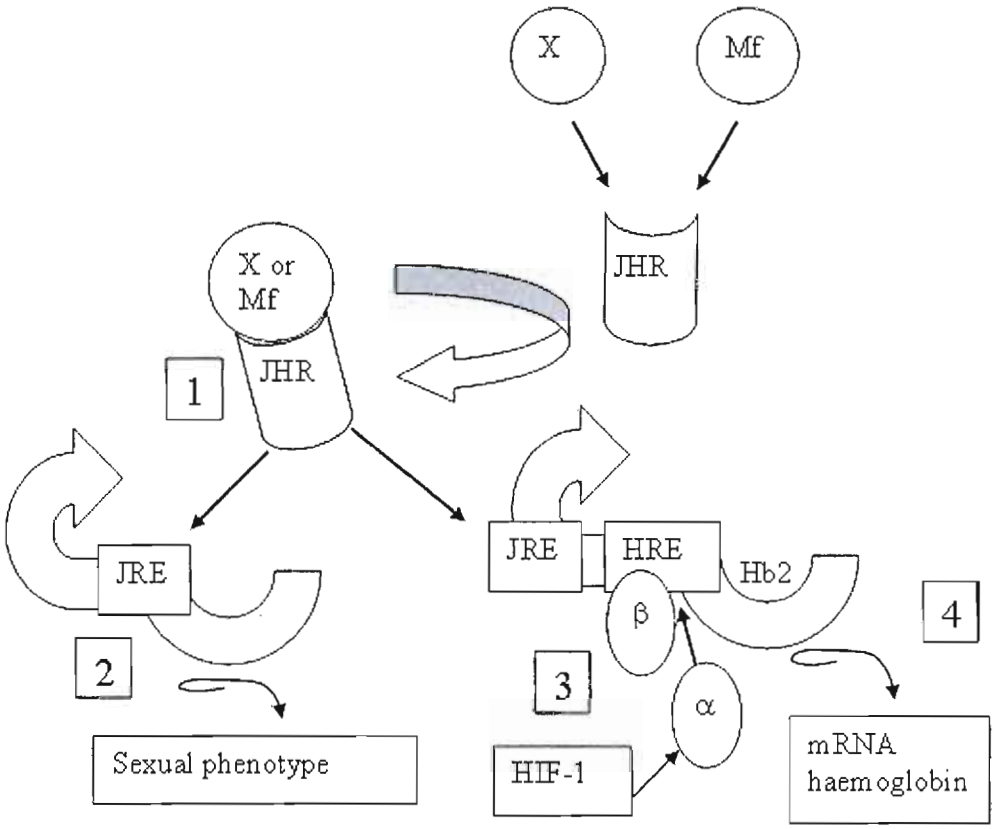
1.Introduction

Many contaminants are known to act as endocrine disrupting chemicals (EDC) at different trophic levels in aquatic ecosystems (Bowerman *et al.*, 2000; Guillette *et al.*, 1994; Tuberty and Mckenney, 2005). In the case of invertebrates, EDC effects at the population level have been documented (Zou and McKeown, 2005) although target mechanisms have only been identified for a few of EDCs (DeFur, 2004; Wu *et al.*, 2004). There is therefore a need for the development of EDC biomarkers and bioassays adapted to aquatics invertebrates (LeBlanc, 2007). One case in point is the lack of specific methods to evaluate the impact of agricultural pesticides that have been designed to act on invertebrate endocrine systems. In addition, certain industrial processes (e.g. pulp and paper production) may be sources of natural plant products that include invertebrate EDCs (Environment Canada, 2002; Martel *et al.*, 2003).

Natural plant products and synthetic chemicals (e.g. certain pesticides) with the capacity to mimic insect juvenile hormones are referred to as juvenoids. Plants are thought to have evolved secondary metabolic products, including terpenoid-based juvenoids, to defend themselves against insects (Bede, 2000; Fowler *et al.*, 2001). For instance, juvabione is found in organic extracts of balsam fir (*Abies balsamea*) (Bowers *et al.*, 1966; Pichette *et al.*, 1998) and acts as a juvenile hormone mimic (Bede, 2000). In fact, juvabione or its precursor has been detected in 3 major tree species (balsam fir, white pine (*Pinus monticola*) and black spruce (*Picea mariana*) (Huber and Bohlmann, 2004) that are used for paper production in Canada (O'Connor *et al.*, 1992). When paper is produced by the thermomechanical pulping (TMP) process, juvabione and other biologically active molecules are extracted and may be constituents of the effluents. Some of these compounds have potent effects on aquatic organisms that could occur through endocrine disruption (Environment Canada, 2002; Martel *et al.*, 1997; Rissanen *et al.*, 1997; Willfor *et al.*, 2004 c). Secondary treatment of pulp and paper mill effluents typically lowers the concentrations of most of the extracted plant products to a significant degree (Martel *et al.*, 1997; Martel *et al.*, 2000). In some cases, with time of exposure and biodegradation, few plant products (ex. phytosterol) have been reported to become more toxic in effluent (Rodden, 2000).

In addition to plant products, there are also some insecticides like fenoxycarb, pyriproxyfen and methoprene that act by mimicking the action of juvenile hormones of many insects and crustaceans (Horst and Walker, 1999; Tatarazako and Oda 2007; Wang *et al.*, 2005). In fact, juvenile hormone analogs are synthesized to control insect pests in agriculture and forestry. A methoprene metabolite is also known to induce the activity of the juvenile hormone of *Daphnia magna* (Wang *et al.*, 2005). Some of those juvenile hormone analogs are known to have effects on reproduction and development of invertebrates like *D. magna* (Templeton and Laufer, 1983).

Daphnia magna reproduce via cyclic parthenogenesis (Weber, 1993) with periods where neonates show only female phenotype. When the environment becomes unfavorable (e.g. diminution of photoperiod), males can be produced (Baer and Owens, 1999). As illustrated in Schema 1 (steps 1 and 2), many environmental signals stimulate the action of the crustacean juvenile hormone, methyl farnesoate (Mf) (Gorr *et al.*, 2006). During ovarian maturation, oocytes exposed to Mf, known as the sexual determinant, will develop the male phenotype (Olmstead and LeBlanc, 2002). Nanomolar concentrations of pesticides that act as juvenile hormone, such as fenoxycarb, pyriproxyfen, methoprene and hydroprene also stimulate production of neonates having male characteristics (Escher and Wahli, 2000; Tatarazako and Oda, 2007). When *Daphnia* are in a sexual reproductive phase, eggs are produced with a protective shell, or epphipia, that can survive desiccation. The F_1 sex ratio is proposed as an endpoint in bioassays of EDC effects in *Daphnia* (Tatarazako and Oda 2007).



Schema 1. Proposed mechanisms for induction of the gene Hb2 coding for haemoglobin (4) and for changes in sexual phenotype (2) in *D. magna* by juvenile hormone methyl farnesoate (Mf) and by juvenile disruptive xenobiotics (X). This mechanism involves an alternative competitive action between two promoter regions (JRE and HRE) of gene Hb2 for haemoglobin production by the complex ligand- juvenile hormone receptor (JHR) (1) and the hypoxia inducible factor-1 (HIF-1) (3). The left side represent the direct action of ligand-JHR on the JRE of the promoter in an unknown gene to regulate changes in sexual phenotype (2)

The juvenile hormone receptor (JHR; Schema 1, step 1) specific to methyl fanesoate has not yet been conclusively identified (Jones and Sharp, 1997; Wozniack *et al.*, 2004). It is considered hypothetically to be a monomeric molecule of 52 kDa and the linking of JHR with the juvenile hormone response element (JRE) at the *cis* position has been clearly demonstrated even for nanomolar concentrations of ligand (Gorr *et al.*, 2006). This JRE consists of a central steroid/retinoid response element and a region rich in 5` AT (Rider *et al.*, 2005).

The gene Hb2 increases the level of haemoglobin in daphnids (Schema 1, step 4; Gorr *et al.*, 2006). There is a direct relationship between haemoglobin increase in female F0 *Daphnia magna* and the occurrence of F1 male neonates. Both responses use the juvenile signalling mechanism in *Daphnia* (Rider *et al.*, 2005). The JRE has been identified for Mf-inducible genes like Hb2. Other sex-determining genes like dmrt, sex and fem might be activated in the same way. In fact, presence of male phenotype is always correlated with haemoglobin increase, but it is possible to observe haemoglobin increase without changes in sexual phenotype.

Gene Hb2 is activated directly through binding of the response element by the complex formed between a juvenoid substance and its receptor (Gorr *et al.*, 2006). The promoter of the Hb2 gene for *Daphnia* contains a response element for hypoxia (HRE) and a JRE that are 10 bp apart and form an alternative response system (Gorr *et al.*, 2004; Gorr *et al.*, 2006). Low levels of oxygen elevate the hypoxia inducible factor (HIF-1) which activates HRE and Hb2 transcription (Schema 1, step 3). When *Daphnia* are not in hypoxia, the gene Hb2 is under the influence of JRE. Mf and pyriproxyfen are two of the rare compounds that have been tested for interacting in this mechanism (Gorr *et al.*, 2006).

The objective of the present investigation was to test if Hb2 gene induction and change in sexual phenotype could be suitable biomarkers for aquatic invertebrates exposed to juvenoid EDCs. Selected pulp/paper mill effluents were examined for their potential juvenoid action. The natural tree product detected in effluents, juvabione, was investigated as a compound potentially contributing to the juvenoid action of effluents. Our hypothesis was that EDCs whose primary action involved JHR binding (i.e. juvenoid hormone mimics) would elicit both Hb2 induction and changes in neonate phenotype (i.e. the production of male neonates). In this context, pyriproxyfen served as a positive control. Alternatively, EDCs might act primarily through abnormal JHR binding (e.g. blockage of JRE-JHR binding) or other signalling mechanisms to produce various related effects like other changes in neonate phenotype or Hb2 induction without the production of male neonates.

2. Materials and methods

2.1. Culture of Daphnia magna

Daphnia magna were cultured for more than 6 months at the Pulp and Paper Research Institute of Canada (PAPRICAN) following Environment Canada protocols (Canadian Pulp and Paper Association/Technical Section, 1992). Basic water characteristics and *Daphnia* maintenance were the same for culture and test conditions (Table 1). The water source was 30% distilled water and 70% filtered well water. Density was (40-50) organisms per litre unless otherwise stated. To maintain the cultures, water was changed once weekly and neonates were removed at least 3 times a week and raised separately. A temperature approximating 20°C or less is required to maintain *Daphnia* in a parthenogenic reproduction phase.

2.2. Effluent and tested chemicals

Effluents were obtained from a newsprint mill in Quebec using the TMP process and using a wood supply of balsam fir and black spruce. The effluents were kept at 4°C during transport and storage. Tests with *Daphnia* were begun within 80 h of effluent collection (with the exception of experiment 3, described below). The effluents were sampled before (three times) and after (four times) secondary treatment in an activated sludge treatment plant. Depending on preliminary results, selected effluents were analyzed for biochemical oxygen demand (BOD) according to standard methods (Canadian Pulp and Paper Association/ Technical section, 1992) as well as resin/fatty acids (RFA) and juvabiones by gas chromatography and mass spectrometry based on the method of Voss and Rapsomatiotis (1985). Other test chemicals were delivered in absolute methanol (less then 0.03 % v/v, final concentration). Test solution and their controls contained the same concentration of methanol. Juvabione was obtained from condensate of balsam fir described by Martel *et al.* (1997). Pyriproxyfen (99% purity) was obtained from Chem Service, West Chester, PA. Preliminary tests were conducted

to select the concentrations of juvabione, pyriproxyfen and effluents that did not cause significant adult mortality after 48-h exposure.

2.3. Test for Hb2 gene induction

Daphnia preserved in 1 ml of Trizol (Life Technology) were homogenized on ice using either a polytron or with a motorized Potter-Elvhein apparatus. A 10-min centrifugation (4°C at 12 000 X g) removed extracellular materiel. The homogenate was incubated for 15 min at 30°C to dissociate the nucleoprotein complex. The different phases were separated with 0.2 ml of chloroform (Sigma) followed by a 15-min centrifugation (4°C at 12 000 X g). RNA was precipitated from the aqueous phase with 0.5 ml of isopropyl alcohol (Sigma-Aldrich). Incubation (10 min) at room temperature was followed by centrifugation (4°C at 12 000 X g for 10 min). RNA was washed with 1 ml of ethanol (75%) and mixed by vortex. Following a 5-min centrifugation (4°C at 7500 X g), the dry pellet was obtained by incubation at 37 °C. The pellet was dissolved in nuclease-free water by a second incubation of 10 min at 60 °C. RNA purity was confirmed by the ratio 260/280 nm using a U.V. spectrophotometer. Then the RNA was transformed to cDNA using a reverse transcription kit (Omniscript RT kit, Qiagen) with a 1 h incubation at 37°C and a 45-min incubation at 42°C. Primers specific for *D. magna* Hb2 gene as described by (Gorr *et al.*, 2006) were synthesized (OPERON) and used for PCR amplification of DNA coding for hemoglobin. DNA amplified using *Daphnia*-specific actin primers (Gorr *et al.*, 2006) served as a reference. Total volume for polymerase chain reaction was 50 µL: 25 µL of PCR master mix (PCR Kit, Qiagen), 2 uL of each primer for sense and anti-sense, 5 µL of cDNA and the rest was filling with nuclease free water. The temperature profile for PCR consists of a denaturizing cycle of 94°C for 3 min, followed by 40 cycles of 1 min of denaturizing at 94°C, 1 min of annealing at 58°C and 1 min for elongation at 72°C. At the end, a final 7 min of elongation was added. PCR products were load in 2 % agarose gel with ethidium bromide. Gels were visualised with U.V. light and band density were quantified with an informatics imagery system (Alphamajeur). The relative hemoglobin quantity was calculated by dividing the band density of Hb2 by the band density of actin.

2.4. Description of 3 different experiments

Experiment 1: Hb2 gene induction

Daphnia, 7 to 9 days old, were exposed to various substances during 24 h. Different concentrations of secondary and primary effluents, juvabione and pyriproxyfen were tested. Each 24-h exposure was conducted with triplicate glass containers of 1 L capacity with a maximal density of 40 *Daphnia*/L. Dissolved oxygen, temperature and pH were monitored at 0 and 24 h for each exposure. At the end of each test, approximately 25-30 *Daphnia* were removed from each container (the remainder were lost during filtration). The combined weight was obtained and the *Daphnia* were rinsed with RNase-free water and transferred to 1 ml of Trizol. Samples were frozen at – 80° in Trizol and kept at this temperature for maximum of 1 year before analysis.

Experiment 2: Neonates phenotype after adult exposure

Adult gravid females ranging from 7 to 14 days of age were exposed individually in 40 ml of test solution in glass containers. Everyday, solutions were changed and *Daphnia* were observed for their neonate production. Experiments were repeated 3 to 10 times. When mortality exceeded 15 % in adult controls, the test was considered to have failed. Neonates from the third broods were transferred to clean water and observed for at least 9 days. However, in the case that the adult female produced only 2 broods after 12 days, the neonates from the second brood were kept for observation.

Experiment 3: Effluent replacement studies

Effluents were kept in a cold room at 4°C and portions used daily in the test for effluent renewal. Before utilisation, effluent temperature was slowly adjusted to 20°C. D.O. and pH were measured everyday. To evaluate potential chemical changes in the effluent during

exposure, containers were either filled once with effluents (i.e. not renewed for the rest of the experiment) or the effluent was changed (i.e. 100% exchange) daily. Comparable controls were run in each test; that is, control water was either renewed daily or not renewed during the course of the exposure. Experiment 1 was repeated as described in section 2.3 however, Hb2 gene induction was tested after a 6-day exposure instead of 24 h. In order to evaluate neonate phenotypes, experiment 2 was repeated as described above with or without effluent renewal.

2.5. Changes in neonate phenotype

The capacity to alter the phenotype of *Daphnia* was examined under conditions that normally favour the production of 100% female neonates. Pyriproxyfen was used as positive control and culture water was used as negative control. Sex of neonates from the third brood was determined by light microscopy (10 X). In order to eliminate the possibility of observation error, neonates were kept for 9 days. *Daphnia* that did not carry eggs or that did not develop normal female morphology after 9 days, were observed and measured under the microscope to determine their sexual status (male or juvenile). Male *Daphnia* are typically smaller with a different shape and can be distinguished by the presence of extended claspers or hooks on the first limb (Hobaek and Larsson, 1990). These criteria for male and female phenotypes were confirmed by morphology of the first antennae. Juvenile phenotype was determined in the present study by a body size smaller than 1.5 mm associated with a lack of egg production and the absence of the male extended claspers. Furthermore, the juvenile phenotype had to be maintained for at least 9 days. The percentage of male, female or juvenile phenotype was calculated based on the total number of live neonates.

2.5. Statistical analysis

Statistical analyses were done with JMP IN 5.1 (SAS Institute 2002). For induction of the

gene Hb2, samples from different experiments were run in the same agarose gel 2%. Data from different treatments and different experiments were compared only when there was no significant difference ($p \geq 0.05$) between their controls (Anova). For treatments with 3 or more replicates, statistical differences between the ratio of Hb2/actine divided by their respective controls were determined using one-way analysis of variance and Dunnett test. For data with less then 3 replicates, they were represented by a graph with the best curve fitting model for induction of the gene link to the different concentrations of the tested substance.

For the changes in sexual status (female, male or juvenile neonate), the Fischer exact test was used. Control as well as identical treatments from different experiments were combined when there was no significant difference between them ($p \geq 0.05$) (Anova). The 4 secondary-treated effluents (A, B, C and D) were considered separately because a significant difference was observed between them in some experiments (Anova, $p < 0.05$).

3. Results

3.1. Induction of the gene Hb2 of Daphnia exposed to different concentration of effluents and juvenoids

When control *Daphnia* were analyzed by reverse transcriptase-PCR, they had a consistent Hb2/actin ratio of 0.55. Compared to controls, pyriproxyfen (1.6 µg/L and 13.8 µg/L) increased the relative expression of gene Hb2 approximately 2-fold (Fig. 1). Hb2 was not significantly induced by 0.06 µg/L of the insecticide. As concentrations of pyriproxyfen increased, the relative expression of Hb2 increased significantly following a logarithmic curve (Fig. 2 C). Efforts to achieve a higher induction were unsuccessful (data not shown). Primary treated effluent elicited significant Hb2 induction even at the least concentrated dilution (10% effluent v/v; Fig. 1) and the response tended to increase with more concentrated primary effluent mixtures (Fig. 2 A). Hb2 induction coincided with a significant decrease in dissolved oxygen occurred at the highest concentration of primary effluent 40%

(v/v), whereas no other effluent condition, either for primary or secondary treatment, was associated with a decrease in ambient oxygen. In the case of secondary treated effluent, however, Hb2 was not significantly induced even at the relatively concentrated mixture (50% v/v). Juvabione significantly increased the relative Hb2 expression at a concentration of 2.4 µg/L (Fig. 1) and elicited maximum Hb2 expression at 7.5 µg/L (Fig. 2 B). In effluent that were analysed, juvabione was detected in all effluent with primary treatment but not, or weakly, detected in effluent with secondary treatment (detection limit 10 µg/L) (Table 2).

3.2. Changes in neonate phenotype

With the positive control pyriproxyfen, the percentage of male neonates increased at the 2 concentrations tested ($p = 0.013$ and $p < 0.0001$) compared to negative control conditions having 100% female neonates (Fig. 3 A). The greater concentration (2.6 µg/L) resulted in 100% male phenotype of the neonates (Fig. 3 C, middle). Exposure to the lower concentration of 0.1 µg/L elicited only few male neonates. A curious observation in these experiments was that all adult females exposed to pyriproxyfen for 24 h developed a posterior 'tail' structure at the location of the dorsal spine which subsequently regressed such that normal female morphology was attained 5 days later (structure not shown).

In contrast to pyriproxyfen, exposure to certain primary or secondary effluents resulted in a "juvenile" phenotype (Fig. 3 C, right) characterized by small size (around 1.5 mm) which was maintained for at least 9 days. Under normal conditions of neonate development, females produced oocytes by 9 days (Fig. 3 C, left) and males developed a sexual appendage referred to as a 'extended claspers or hooks used to hold the female. In contrast, the juvenile phenotype had neither oocytes nor extended claspers and their body size remained small. Primary effluent (2.5% v/v) and secondary effluent sample D (50% v/v) caused an increase of the juvenile phenotype (Fig. 3 B, D). Samples A, B and C of secondary effluent, however, did not induce the juvenile phenotype (Fig. 3 D). Exposure to juvabione at a concentration of 2.25 µg/L and higher concentrations completely inhibited egg development (data not shown)

such that sexual phenotypes of neonates could not be assessed.

3.3. Effects of effluent renewal and time of exposure on Hb2 gene induction and neonate phenotype

As a preliminary test to examine whether time of exposure and renewal of effluent influenced Hb2 expression, secondary treated effluent A was first evaluated. *Daphnia* were held for 6 days with or without daily renewal of the control water. In controls, a slight but significant increase ($p < 0.05$) in Hb2 expression occurred under the non-renewal control conditions (Fig. 4 A) while dissolved oxygen was only around 0.5 mg/L lower after 6 days (Fig. 4 B). In the case of secondary treated effluent with daily renewal, however, dissolved oxygen at day 6 decreased significantly compared to the renewed control water (Fig. 4 B). Hb2 induction was also significantly greater in *Daphnia* exposed to the secondary effluent (40% v/v) with daily renewal than in the group with renewal of control water (Fig. 4 A). When *Daphnia* were exposed to the same effluent but without renewal, neither Hb2 nor ambient oxygen was significantly affected (relative to non-renewed control group).

Daphnia exposed for 6 days to 40 % v/v of secondary treated effluent A without renewal showed no changes in neonate phenotype compared to controls (Fig. 5). Similarly, exposure to the same effluent with daily renewal did not affect neonate phenotype (Fig. 3 D). In contrast, 6-day exposures to 20% v/v of the secondary treated effluent sample C or sample D without daily renewal resulted in 18% or 9% males, respectively, in the third brood (Fig. 5). Furthermore, effluent sample D without renewal elicited the juvenile phenotype in 9% of neonates (Fig. 5). Under identical experimental conditions, but with daily renewal of effluents C, only female neonates were produced (Fig. 3 D).

A preliminary experiment was conducted to assess the reproductive ability of males produced during pyriproxyfen and effluent exposures. Groups of 5 males and 5 unexposed females were placed together. In the case of males produced during pyriproxyfen exposure,

apparently normal copulation was observed but no epphipia developed in the females. In the case of males produced during exposure to secondary effluent samples C and D, however, epphipias were observed in females after 8 days as occurs during sexual reproduction.

4. Discussion

Within the broad category of environmental contaminants that potentially affect endocrine systems are certain insecticides that mimic the action of juvenile hormones. In *Daphnia magna*, such "juvenoids" are expected to bind JHR and thus cause the production of male offspring. In addition, JHR binding initiates events leading to Hb2 transcription (under conditions of oxygen sufficiency). The fact that the crustacean JHR has not been identified precludes direct investigation of juvenoid-JHR binding (Fang *et al.* 2005; Jones and Sharp, 1997). In the present experiments therefore, Hb2 induction and the male phenotype of neonates were taken to be criteria for activation of the juvenile hormone signalling pathway.

In the present experiments, pyriproxyfen induced Hb2 (Fig. 1, 2) and caused the production of neonates with the male phenotype (Fig. 3 A). The growth-regulating insecticide pyriproxyfen has previously been identified as a juvenile hormone agonist (LeBlanc, 2007; Gorr *et al.*, 2006; Tatarazako and Oda 2007). The results of the present investigation therefore confirmed the mode of action of this pesticide. More specifically, the low pyriproxyfen concentration (0.1μg/L) produced mixed broods (Fig. 3 A) and the same concentration of pyriproxyfen was found to be the threshold for mixed broods in *Daphnia* (Table 3; Tatarazako and Oda, 2007).

Effluent samples obtained from a thermomechanical pulping process after primary treatment caused a significant, concentration-dependent increase in Hb2 transcription (Fig. 1, 2). Ambient oxygen remained near saturation during the 24h-effluent exposures (except at 40% v/v) such that Hb2 induction was not attributed to chemical oxygen demand. However, the primary effluent failed to cause the production of male neonates as expected in the case of a

juvenile hormone agonist. Instead, relatively low concentrations of the primary effluent elicited neonates having a "juvenile" phenotype (Fig. 3 B) possibly related to abnormal juvenile hormone signalling (Schema 1, steps 1 and 2). It is interesting to note the parallel between the juvenile phenotype observed in the present study and the action of the juvenile hormones in preventing maturity (i.e. prolonging juvenile stages) in many groups of invertebrates (Romeo, 2005; Zaoral and Slama, 1970). Juvabione is a constituent found in some primary mill effluents (Martel *et al.*, 1997) and is known to act as a juvenile analog (Bede, 2000). Its presence in primary effluents of the present investigation was confirmed by GC/MS (Table 2). Except at the highest juvabione concentration, Hb2 induction increased in a concentration-dependent manner (Fig. 2 B). This exception can be partially explained by the possible difference between biologically available concentration of juvabione and the nominal concentration due to the difficulties to effectively solubilize juvabione.

The experiment on effluent renewal during 6-day exposures highlights the close link between hypoxia and induction of the gene Hb2 by the HIF-1/HRE pathways (Schema 1 step 3). A decrease in oxygen levels below 5 mg/L coincided with a 1.4-fold Hb2 induction in *Daphnia* exposed to effluent A with daily renewal (Fig. 4). This result may be explained by hypoxic induction of the gene Hb2. Comparison of the two controls, i.e. with or without renewal, further suggests that even a 0.5 mg/L reduction in ambient oxygen level maintained for 6 days may have caused the 1.3-fold Hb2 induction (Fig. 4). At optimal concentrations, our data for Hb2 induction by juvenoids was consistently greater than 2-fold, indicating a more pronounced signalling though the JHR/JRE system compared with hypoxic induction through the oxygen sensing system HIF-1/HRE (Schema 1 step 3).

Previous studies demonstrated that Hb2 induction by juvenoids coincides with changes in the phenotype of neonates (Rider *et al.* 2005). However, in the present experiments, none of the effluent exposures elicited both Hb2 induction and the male phenotype. The response to effluents was therefore not considered to be consistent with the criteria for activation of the juvenile hormone signalling pathway. Instead of the typical male phenotype, the effluents were associated with a "juvenile" phenotype which constituted 80% or 100% of neonates in

some cases (Fig. 3 B, D). Similarly, in the case of arthropods exposed to juvenoids, many authors have reported larvae with juvenile characteristics which LeBlanc *et al.* (1999) proposed as a biomarker of endocrine disruption due to its rare occurrence and specificity. This type of effect has not been reported previously for *Daphnia* but is known to occur in other crustaceans (Abdu *et al.*, 1998; Verslycke *et al.*, 2004).

A curious observation in the present study was the production of phenotypic male neonates following pyriproxyfen exposure which were unable to reproduce sexually. Certain juvenile hormone analogs are capable of arresting spermatozoid production in invertebrates (Salehzadeh *et al.*, 2003; Skattebol *et al.*, 2004). It is possible that pyriproxyfen inhibited spermatozoid production in the present study. The lack of sexual reproduction may also be related to possible behavioural, anatomical or endocrine effects on the male system.

Juvabione is able to elicit both male and juvenile phenotypes in insects (Bede, 2000; Whittaker and Feeny, 1971). Primary effluents supposedly containing juvabione (Table 2) also caused the production of the juvenile phenotype in the present study (Fig. 3 B). In contrast, secondary effluent contained very low ($\leq$ 0.04 mg/L) concentrations of juvabione and did not elicit the juvenile phenotype (Table 2, Fig. 3). Chemical analysis for juvabione was not conducted in the case of effluent D and no conclusion can be made as for the role of juvabione in this effluent. Previous studies have also reported that juvabione was detected in primary treated effluents from thermomechanical pulp mills but was partially removed by secondary treatment (Kantardjieff *et al.*, 1990; Martel *et al.*, 1997). One hypothesis to explain the coexistence of those phenotypes with juvenoid substances is the interaction between Mf signalling and with other endocrine systems (e.g. ecdysteroids). Many studies have demonstrated mechanistic links between juvenile hormone analogs and possible anti-ecdysteroids effects (Antoniewski *et al.*, 1996; Fang *et al.*, 2005; Ghbeish *et al.*, 2001; Mu and LeBlanc, 2002; Mu and LeBlanc, 2004; Wu *et al.*, 2004). Table 3 is a summary of different *Daphnia* sexual phenotypes and putative actions reported after exposure to chemicals or effluents.

In summary, the results of the present study demonstrate that primary treated effluents from thermomechanical pulp mills activate the juvenile hormone signalling pathway (Hb2) and produce a juvenile phenotype in *Daphnia*. These effects may be associated with the presence of juvabione. In three out of four secondary treated effluents there was no indication that the juvenile hormone signalling was activated.

Acknowledgments

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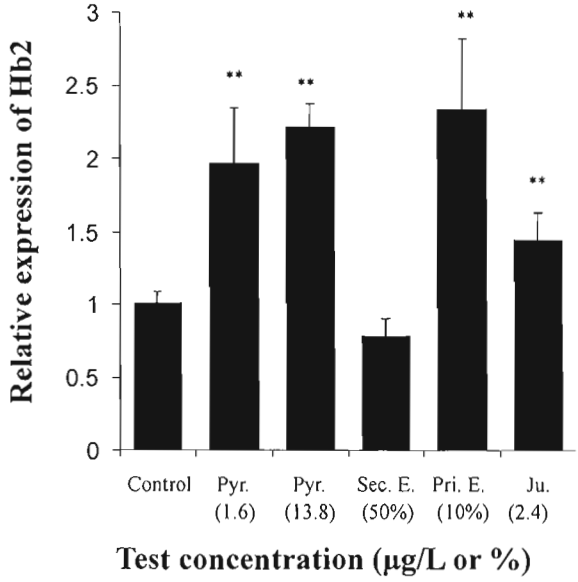

Figure 1. Relative expression of the gene Hb2 ((Hb2/actine)/control) from *D. magna* exposed (24 h) to Pyr. (Pyriproxifen), Sec. E. (Secondary effluent), Pri. E. (Primary effluent) and Ju. (Juvabione). Bars indicate standard error of the mean, for control (n = 7) and for all the others (n = 3). The asterisk indicates statistically significant difference between the substance exposed and the control

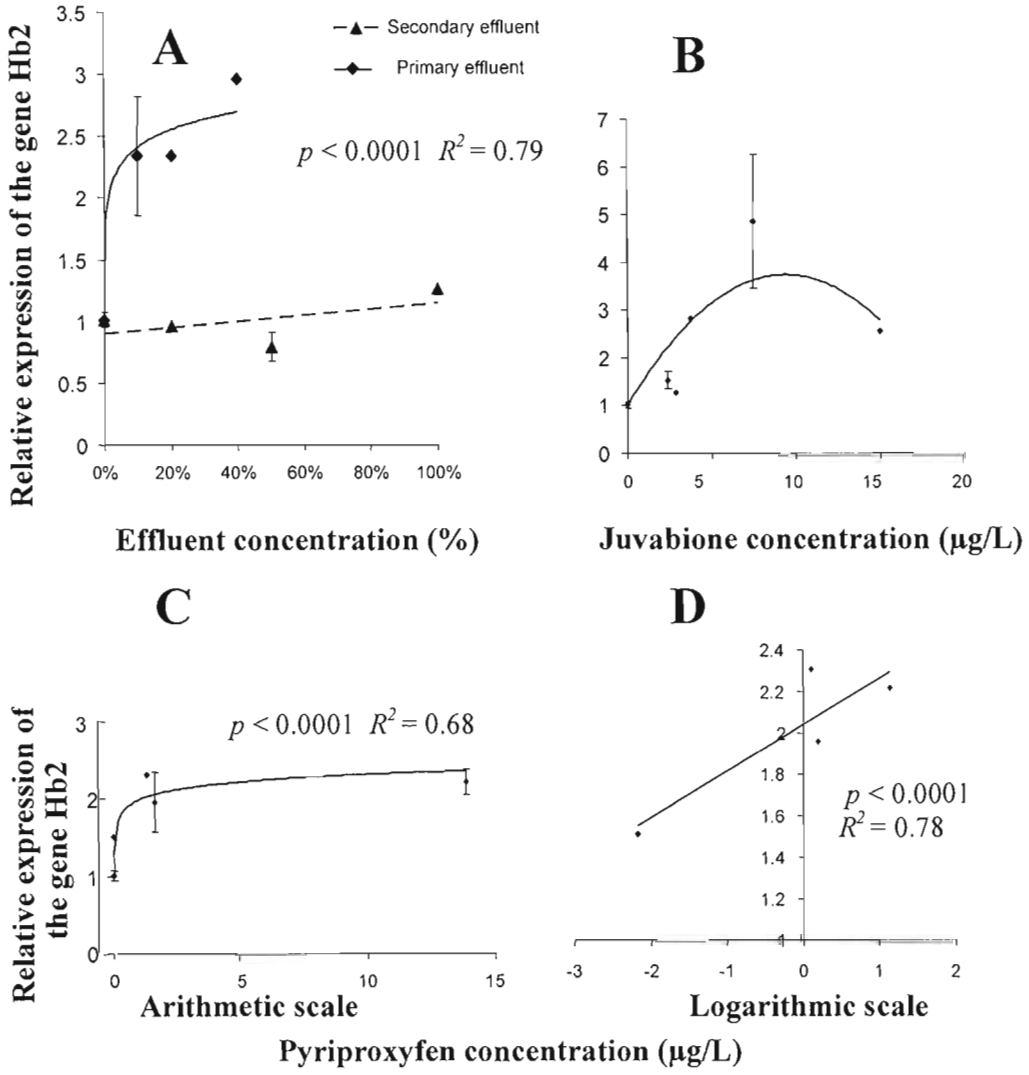

Figure 2. Relative expression of the gene hb2 in *Daphnia magna* exposed 24 h to different concentrations of (A) primary and secondary treated effluents (B) juvabione and (C) pyriproxyfen arithmetic scale (D) pyriproxyfen logarithmic scale. For juvabione and secondary treated effluents, no statistical test was performed because there was no homogeneity and/or normality of residuals. (A) For secondary treated effluents, n = 12, and for primary treated effluents, n = 12. For juvabione (B), n = 16. For pyriproxyfen (C), n = 16 and (D) n = 16.

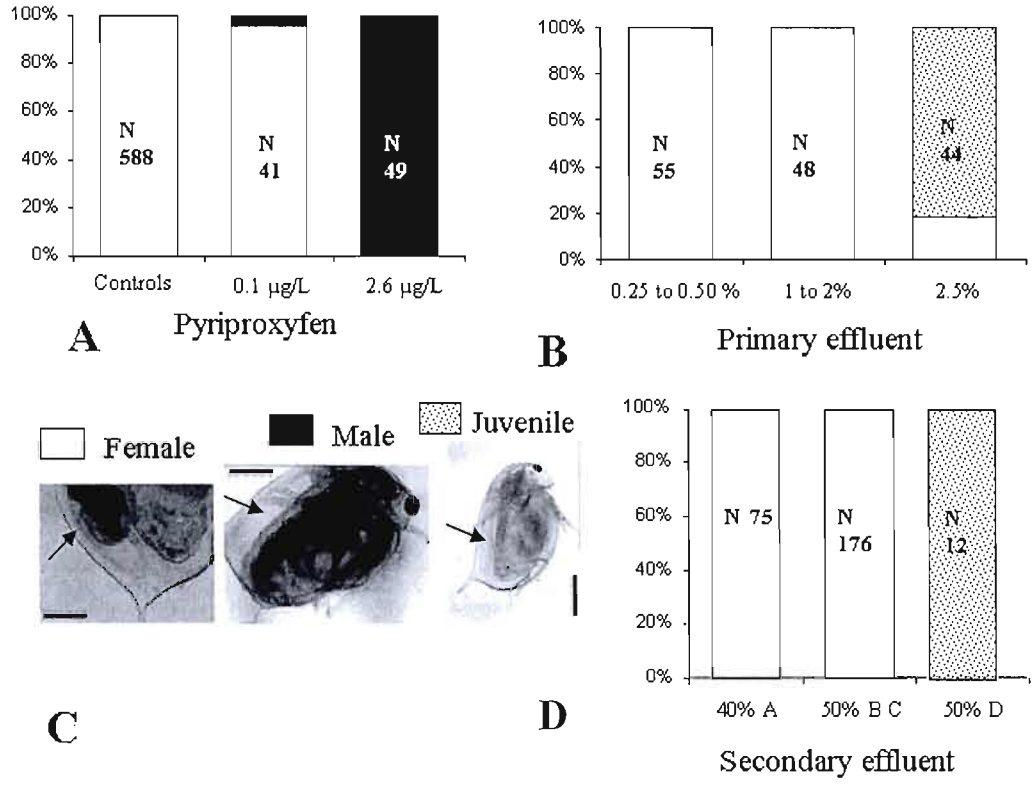

Figure 3. Percentage of sexual phenotype of the third brood of *D. magna* with prenatal expositions to (A) pyriproxyfen (B) primary effluents and (D) secondary effluents. For each treatments the number of replicas varies between 3 and 36 dues to mortality. (C) is the legend with picture of 9 days old *Daphnia* grown in clear water (from left to right) : female from control, male from pyriproxyfen and juvenile from primary treated effluent. Arrows represent the brood chamber with embryos in the female, but empty in the male and the juvenile. The scale for each picture is a black line that represent 500 µm. Pearson test for the distribution of sexual ratio of *D. magna* exposed to pyriproxyfen (0.1µg/L) compared to control is Chi^2 = 6.15, DF = 41, p = 0.013 and for pyriproxyfen (2.6 µg/L) compared to control is Chi^2 = 46, DF = 45, $p < 0.0001$

Note. No neonate were observed for the third brood with juvabione

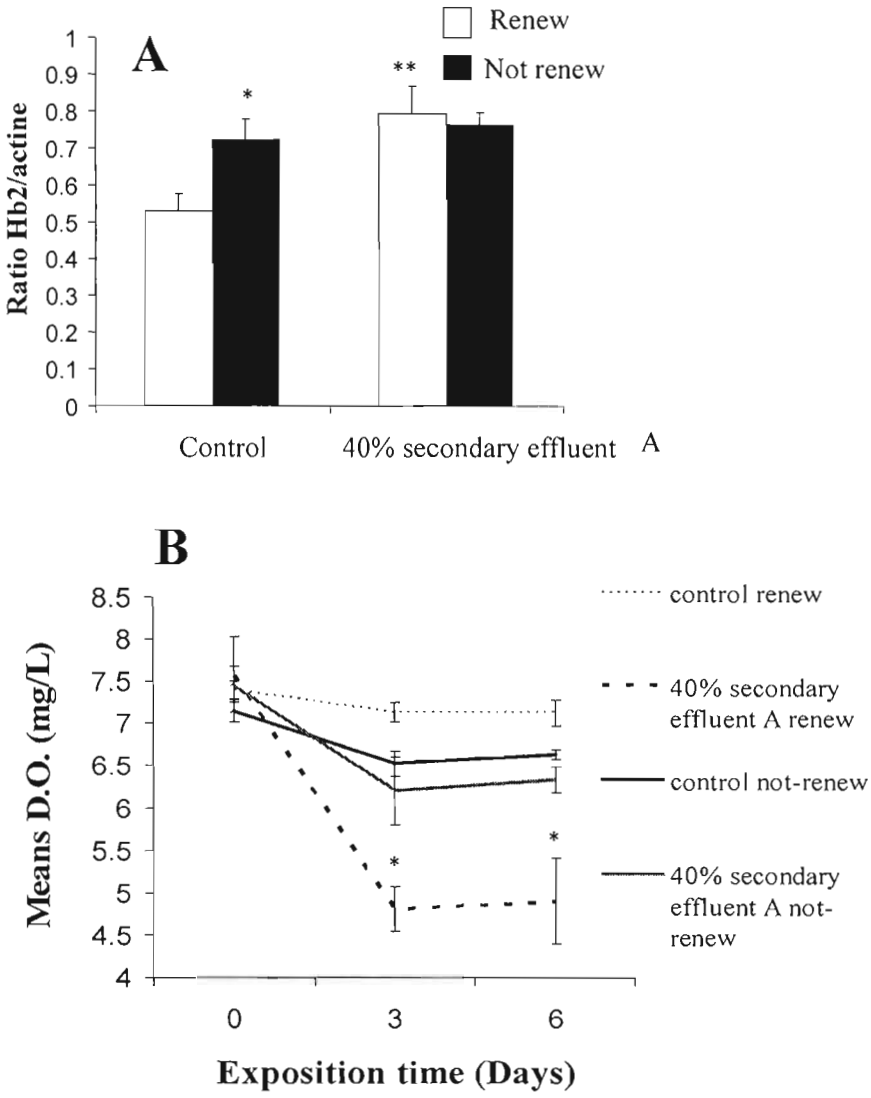

Figure 4. (A) Relative expression of the gene Hb2 ((Hb2/actine)/control) from *D. magna* exposed (6 days) to 40% secondary treated effluent A renew or not renew. (B)Mean dissolve oxygen of control and 40% (v/v) secondary treated effluent A measured at different days. For (A) and (B) bars indicate standard error of the mean (n = 3). For (A) and (B), two asterisks indicate statistically significant difference between the controls. One asterisk indicates statistically significant difference between the substance exposed and the control

Note. Secondary effluent A seems to have some characteristics of a biologically active effluent i.e. DO was lower when the effluent was renewed. DO of effluent A renewed (40% v/v) did not meet the level presented in table 1 (see [b]). Effluent A has a BOD of 23 mg/L

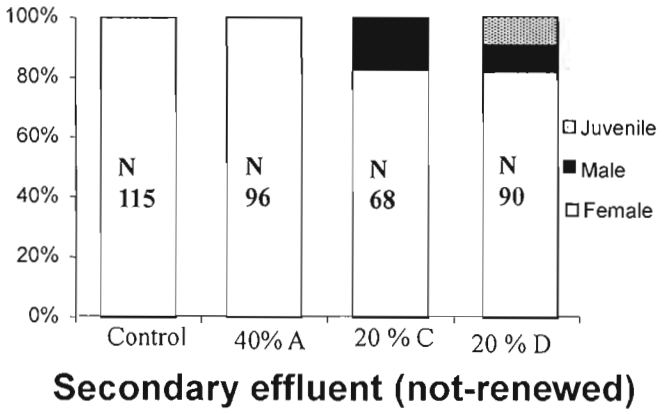

Figure 5. Sexual phenotype (%) of neonates from the third brood, from *Daphnia* continuously exposed to secondary treated effluent or to control *

**Note.* The control used to test effluent A was not renewed, however, for effluent C and D the control was renewed (same control as Fig. 3)

Table 1. Basic water and maintenance conditions[a] for *D. magna*.

Parameters	Frequency	Measure
Temperature	Constant	20-22°C
pH	Weekly	7±2
Dissolved oxygen[b]	Weekly	6-8 mg/L
Conductivity	Weekly	1050-1200 µmhos
Light intensity	Weekly	400-800 lux
Photoperiod day/night		16L : 8D
Regimen - 1L	Daily	5 ml algae *Selenastrum capricornutum* for 2 ml YCT (yeast cerophyll-trout chow)
Regimen - 40 ml	Daily	0.4 ml algae for 0.2 ml YCT

[a] Complete water quality parameters are found in Kovacs and Voss (1992)
[b] Except for certain experimental conditions presented in the text

Table 2. Chemical analysis for RFA (resin and fatty acid), BOD (biochemical oxygen demand), juvabione and dehydrojuvabione in effluents. Same letter represent same effluent. The asterisk (*) indicates effluent not tested in this study, but obtained from the same mill with the same treatment plants and the same sampling methods that the one used in this study. D = détected, ND = not detected (detection limit 10 µg/L).

Effluent	Treatments	RFA (mg/L)	BOD (mg/L)	Juvabione (mg/L)	Dehydro Juvabione (mg/L)
A	Secondary	0.23	23	0.02	0.02
B	Primary				
B	Secondary	0.02		ND	ND
C	Secondary		8		
D	Primary				
D	Secondary				
E	Primary			D	D
***F**	Primary	11.4		6.44	1.06
***F**	Secondary	0.05		0.04	ND
***G**	Primary	25.4		8.9	5.9
***G**	Secondary	0.1		ND	ND
***H**	Primary	19.84		13.15	3.98
***H**	Secondary	0.08		0.02	0.01

Table 3. Different sexual phenotype of neonate *Daphnia magna*[a] and putative action.

Substances	Putative action	Observation	References
Pyriproxyfen 0.1 µg/L	Juvenoids	Male and female	Present study Tatarazako and Oda 2007
Pyriproxyfen 2.6 µg/L	Juvenoids	Male no sexual reproduction	Present study
Pyriproxyfen 1 µg/L	Juvenoids	Male	LeBlanc 2007
Primary effluent 2.5% (v/v)	Juvenoids and anti-ecdysteroid	Female and juvenile	Present study
Secondary effluent A,B,C	None	Female	Present study
Secondary effluent not-renew A	None	Female	Present study
All controls	None	Female	Present study
Secondary effluent D	Unknown	Juvenile	Present study
Secondary effluent not-renew C	Metabolic toxicity	Male and female	Present study
Secondary effluent not-renew D	Metabolic toxicity	Male, female and juvenile	Present study
Juvabione 2.25 µg/L	Juvenoids and anti-ecdysteroids	No eggs	Present study
Juvabione	Unknown	Juvenile and sterile male (insects) [a]	Whittaker and Feeny 1971

[a] Except otherwise stated

ARTICLE II

POTENTIAL JUVENILE AND ECDYSTEROID DISRUPTING EFFECTS OF PULP MILL EFFLUENTS AND JUVABIONE IN *DAPHNIA MAGNA*

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Abstract : Certain growth-regulating insecticides are known to affect the endocrine systems of invertebrates, particularly the juvenile and ecdysteroidal hormonal systems. Trees have evolved secondary products, e.g. juvabione, having insect hormonal properties and these may be liberated in the thermomechanical preparation of pulp. The main objective of the present research is the detection of biological effects of potential juvenile and/or ecdysteroid disruptors in pulp mill effluents. Tests were done with 5 biological indicators on adult *Daphnia magna* and their progeny. Moulting time under the control of ecdysteroids and brood sizes, were the parameters selected for adults. For their progeny, changes in sexual phenotype, size and mortality were measured. With the juvenile hormone analog pyriproxyfen (2.6 µg/L) a reduction in brood size and an increase in moulting time were observed. All neonates demonstrated the male phenotype. Exposure of adult *Daphnia* to 2.5% (v/v) primary treated pulp effluent from a thermomechanical pulp mill, resulted in a decrease the number of neonates per brood and an increase of moulting time. Neonates were significantly smaller and remained sexually immature. Juvabione, completely inhibited *Daphnia* reproduction at a concentration of 2.25 µg/L and delayed moulting. For *Daphnia* exposed to a series of secondary treated effluents at a dilution of 50% (v/v), 3 effluents out of 4 did not affect the number of neonates per brood, neonate body size, or the moulting time. Juvabione concentrations were considerably reduced by secondary treatment. The results suggest that primary treated pulp mill effluents and the natural tree product juvabione interfere with juvenoid and ecdysteroid signalling. These effects are mostly eliminated by secondary treatment of mill effluents.

Keywords: Bioindicator, Pulp and paper effluent, Moult, Growth, Sexual phenotype

1. Introduction

Invertebrates can adapt to their environment through the perception of external cues that activate neuro-endocrine regulation of a variety of key physiological processes. Thus, hormones like ecdysteroids and juvenile hormones can have major effects on invertebrate phenotype (e.g. sex, size) and fitness (e.g. mode of reproduction, brood sizes, survival). It is suggested that substances which interfere with central endocrine system signalling pathways can also have important impacts on crustacean populations (Zou and McKeown, 2005).

The juvenile hormone of crustaceans, methyl farnesoate (Mf), controls metamorphosis (e.g. morphogenesis of larva in *D. magna*) and is implicated in reproductive maturation (secondary sexual characteristics) and male *Daphnia* behaviour (Fingerman, 1997). For the temporary parthenogenic *D. magna*, Mf is the sexual determinant; oocytes exposed to this hormone during the ovarian maturation develop the male phenotype (LeBlanc *et al.*, 1999; Olmstead and LeBlanc, 2002). Substances able to interfere or mimic juvenile hormone are called juvenile hormone analogs. Presently, only a select group of growth-regulating insecticides has been identified as juvenile hormone agonists affecting crustaceans (LeBlanc 2007; Tatarazako and Oda, 2007). Juvenile hormone analogs can inhibit egg development in some insects (Bede, 2000; Huber and Bohlmann, 2004; Salehzadeh *et al.*, 2003) and impair reproduction and development of *Daphnia magna* (Templeton and Laufer, 1983), the blue crab *Callinectes sapidus* (Horst and Walker, 1999) and the estuarine mysid *Mysidopsis bahia* (McKenney and Celestial, 1996).

Ecdysteroids play a crucial role in *Daphnia* populations by regulating the moulting process (Quackenbush, 1986) thus determining growth and egg production in *D. magna* (LeBlanc, 2007). Ecdysteroids are also necessary for normal embryonic development and reproduction (Chang *et al.*, 2001). The fungicides propiconazole and fenarimol inhibit enzymes of the ecdysterone synthetic pathway (Ronis *et al.*, 1998; Williams *et al.*, 2000) while ecdysterone agonistic effects have been reported for the plant product ponasterone A and the insecticide RH 5849 (Baldwin *et al.*, 2001; Clare *et al.*, 1992; LeBlanc, 2007). Interference with

ecdysteroid receptor binding has been demonstrated for several organochlorine insecticides, the industrial chemicals bisphenol A, diethylphthalate and 4-nonylphenol as well as the natural products reseratrol and zearalenone (LeBlanc, 2007). Juvenile hormone analogs might also have ecdysteroid disruptor activities (Mu and LeBlanc, 2004; Tuberty and McKenney, 2005), however, few studies have investigated the interactions between perturbation of juvenile hormone and ecdysteroid signalling in crustaceans (LeBlanc, 2007).

Plants have evolved a diversity of secondary metabolic products including certain terpenoid-based compounds that are induced by insect attack and act as juvenile hormone analogs to inhibit insect maturation (Romeo, 2005). Two of these juvenile hormone analogs, juvabione and dehydrojuvabione, are natural products of the balsam fir (*Abies balsamea*) (Bowers *et al.*, 1966; Pichette *et al.*, 1998), one of the principal species treated by thermomechanical processes in Québec paper mills (O'Connor *et al.*, 1992). In tree extracts, epimanool is a natural substance often associate with juvabione (Martel *et al.*, 1997). Other natural plant product groups extracted by thermomechanical processes include resin acids, phytosterols, stilbenes, flavinoids, polyphenols and fatty acids (O'Connor *et al.*, 1992; Pranovich *et al.* 2005; Verenich *et al.*, 2004). Secondary treatment of effluents includes the utilisation of micro-organisms to accelerate natural decomposition of organic wastes by activation of organic residues or aeration stabilization (Environment Quebec, 2002). This also results in the removal of large proportions of terpenoid-based juvabione and other natural tree chemicals (Martel *et al.,* 1997). Ozonation is another treatment that could reduce those substances (Roy-Arcand, 1996; Pockhrel and Viraraghavan, 2004). Effluent replacements have also been reported to influence toxicity of effluents (Rodden, 2000).

The objective of this study is to assess the capacities of different treatments of pulp and paper mill effluents and natural tree chemicals (i.e. epimanool and juvabione) to elicit typical juvenile hormone and ecdysteroid responses. Pyriproxyfen, a well-documented juvenile analog, served as a positive control. A second objective is to evaluate biological indicators potentially useful for aquatic invertebrate bioassays.

2. Materials and methods

2.1. Culture of Daphnia magna

D. magna were cultured for more than 6 months at the Pulp and Paper Research Institute of Canada (Paprican; Pointe-Claire, Canada) that follow Environment Canada protocols (Canadian Pulp and Paper Association/ Technical section, 1992). Basic water characteristics and *Daphnia* maintenance were the same for culture and test conditions (Table 1). Density was (30-40) organisms per litre unless otherwise stated. To maintain the cultures, water was changed once weekly and neonates were removed at least 3 times a week and raised separately. A temperature approximating 20°C or less is required to maintain *Daphnia* in a parthenogenic reproduction phase. Dead *Daphnia* were recorded and moult skins were removed.

2.2. Effluent and test chemicals

The effluents were obtained from a newsprint mill in Québec, Canada, which uses a thermomechanical process (TMP) to treat balsam fir and black spruce. The effluents were kept at 4°C during transport and storage. The test with *Daphnia* began within 80 h of effluent collection (with the exception of experiment 3, described below). Three primary treated effluents and four secondary effluents treated by activated sludge were examined. In addition, three other effluents with different ozone treatments (effluent 1: 30 mg/dm^3, effluent 2 : 100 mg/dm^3 and effluent 3 : 300 mg/dm^3) were randomly sampled at the pulp mills. Effluents were analyzed for biochemical oxygen demand (BOD) according to standard methods (Canadian Pulp and Paper Association/ Technical section, 1992) and resin /fatty acids (RFA) and juvabiones by gas chromatography and mass spectrometry (Voss and Rapsomatiotis, 1985).

Other than effluents, test chemicals were dissolved in absolute methanol and diluted to less

then 0.03 % v/v in the final concentration. Controls contained the same concentration of methanol. Juvabione was obtained from condensate of balsam fir as described by Martel *et al.*, 1997. A mixture of (49%) juvabione and (43%) epimanool was also tested. Pyriproxyfen (99% purity) was obtained from Chem Service, West Chester, PA. The 48h LC50 was measured using 7 day-old *D. magna* as preliminary tests to select the concentrations of juvabione, pyriproxyfen and effluents that did not cause significant adult mortality after 48h exposure.

2.3. *Daphnia* experiments

Experiment 1: Brood size and moulting time of exposed adult *D. magna*

Gravid females ranging from 7 to 14 days of age were individually exposed to 40 ml of test solution in glass containers. Experiments were repeated 3 to 10 times. Everyday, solutions were changed and the number of newborn *Daphnia* was counted. The neonates from a given brood were transferred to a separate jar to follow the life of the progeny. Brood size was calculated as the mean of neonates produced in the 2^{nd} and 3^{rd} broods for each adult female *Daphnia*. In the case that the exposed female *Daphnia* survived more than 12 days but second and third broods were not produced, the mean brood size was taken to be 0. As moulting coincides with laying, moulting time was estimated by the time between broods for female *D. magna*. Diminished moulting time is a method used to detect anti-ecdysteroidal activity in *Daphnia* (Baldwin *et al.*, 2001).

Experiment 2: Neonate sexual phenotype, body size and mortality after adult exposure

Neonates obtained from the third broods of experiment 1, were transferred to clean water and observed for sexual phenotype, size and mortality. However, when an adult female produced only 2 broods after 12 days, the neonates from the second brood were kept for observation. The determination of sexual phenotype is described in the section 2.4. Sizes of neonates were

measured with a microscope (10X) after 8 and/or 15 days. Each *D. magna* was placed in a drop of water on a glass slide, immobilised by removing the water and placed laterally. The body length was measured from the point immediately above the eyespot to the base of the dorsal spine (Johnson and Delaney, 1998). Percentage of mortality of neonates from the third broods was calculated after 8 days.

To evaluate the effects of subsequent juvenile hormone analog exposure on mortality, mortality rate was measured in control exposure in neonate, in pyriproxyfen pre-natal and post-natal exposures.

Experiment 3: Secondary treated effluent replacement studies

Effluents were kept in a cold room at 4°C and portions were used daily for effluent replacement study. Before utilization, effluent temperature was slowly adjusted to 20°C. D.O. and pH were measured everyday. To evaluate effects of potential chemical changes in the effluent during exposure, experiments 1 and 2 were conducted with and without effluent replacement. Containers were either filled once with effluents (i.e. not renewed for the rest of the experiment) or the effluent was changed (i.e. 100% exchange) daily. Comparable controls were run in each test; that is, control water was either renewed daily or not renewed during the course of the exposure.

2.4. Changes in sexual phenotype of neonates

The capacity of effluents and test chemicals to alter the sexual phenotype of *Daphnia* was examined under conditions that normally favour the production of 100% female neonates (Table 1). Pyriproxyfen was used as positive control and culture water was used as negative control. Sex of neonates from the third brood was determined by light microscopy (10 X). Whereas, males can be differentiated from females after only 24h by the length and morphology of the first antennae (Tatarazako and Oda, 2007), in order to eliminate the

possibility of observation error, neonates were kept for 9 days in the present study (Fig. 1 A, B). After this period, *D.magna* which neither carried eggs nor developed normal female morphology were observed and measured to determine their sexual status. Mature male *Daphnia* are typically smaller with a different shape and can be distinguished by the presence of extended claspers or hooks on the first limb to hold the female (Hobaek and Larsson, 1990). The percentage of each sexual phenotype was calculated based on the total number of live neonates.

2.5. Data analysis

Statistical analysis was done using JMP IN 5.1 (SAS Institute 2001). For each experiment, when mortality exceeded 15 % in adult controls, the test was considered to have failed. Significant differences between treatments and controls for brood size, survival and body length were evaluated ether with a Student's T test or with Anova followed by the Dunnett test. When there was no significant difference between identical treatments from different experiments, data were combined (Anova, $p \geq 0.05$). To analyse the impact of primary effluent concentrations on brood size, the best curve fitting model was used. For difference in number of broods per day, the Wilcoxon test was used.

The 4 secondary-treated effluents (A, B, C and D) collected at different times were considered separately because a significant difference was observed between them in some experiments (Anova, $p < 0.05$). In the case of primary effluents, all experiments were conducted using the 3 effluent samples. Results were never found to be significantly different with the 3 primary treated effluents at the same concentration and therefore the results were combined. In the preliminary LC50 test, however, only one of the primary treated effluents was used.

3. Results

LC50 was attained in a system containing approximately 15% of the primary effluent tested and 7.5 µg/L for juvabione (Fig. 2). No mortality of adults was observed with exposure to 2.5% primary effluent and 2.25 µg/L juvabione. With control, pyriproxyfen (2.6 and 0.1 µg/L), and secondary treated effluent (20%, 40% and 50%), the mortality rate after 48h, was less then 5 % (data not shown).

3.1. Broods size and moulting time of exposed adult D. magna

When *D.magna* were exposed to the juvenile hormone analog pyriproxyfen (2.6 µg/L), the mean number of neonates per brood was 3 times lower than in the control group (17.5 ±0.9) (Fig. 3). Secondary treated effluent D (50% v/v) and the randomly sampled effluent 1 (ozone treatment 30 mg/dm^3 at 50% v/v) also significantly decreased the mean number of neonates per brood. When *Daphnia* were exposed to 0.5 % to 5% primary effluents, the brood sizes were significantly less than control broods, the numbers decreasing logarithmically (Fig. 4). No neonates were observed following exposure to juvabione (2.25 µg/L) or a mixture of 49% juvabione and 41% epimanool (2.25 µg/L). With *D. magna* exposed to either 5% primary treated effluent or to juvabione (0.225 µg/L), the neonates did not survive (data not shown).

The number of broods per day was approximately 0.3 ± 0.06 for the control group. However, the juvenile hormone analogs (2.6 µg/L), as well as juvabione (2.25 µg/L), the mixture of epimannol and juvabione, effluent 1 (50% v/v) and primary treated effluent (1 to 2.5% v/v) lead to a decrease in the mean number of broods per day (Fig. 5). Even at a lower juvabione concentration (0.225 µg/L), a diminution of the mean broods per day was found (data not shown). Generally, the mean broods per day decreased when concentrations of pyriproxyfen, primary effluent and juvabione increased.

3.2. Neonate phenotype, body size and mortality after adult exposure

Neonates developed the sexual phenotype characteristic of males or females after different adult exposures. Pyriproxyfen at the highest concentration (2.6 µg/L) increased the percentage of male neonates, with 100% change of sexual phenotype, compared to control neonates (Fig. 6). At 0.1 µg/L, only a few neonate males were observed. Males produced by adults exposed to pyriproxyfen were tested for their capacity to reproduce sexually by placing 5 males and 5 control females together. Although copulation was observed, no epphipia (diapause resting eggs) developed in the females. Instead, after 8 days, all females produced neonates. The same experiment was conducted with 5 males obtained from effluent D (20 % v/v; without daily renewal). In this case, copulation was observed and epphipia developed in all females.

Primary effluent (2.5% v/v) and secondary effluent D (50% v/v) caused a "juvenile" phenotype. With *Daphnia* exposed to primary effluent, around 80% of *Daphnia* had the phenotype "juvenile" instead of the normal female phenotype (Fig. 6). In the present study, juvenile phenotype was defined by a body size smaller than 1.5 mm maintained for at least 9 days (Fig. 1 C), associated with a lack of egg production.

Neonate survival depended on the substance to which adult females had been exposed. More than 80% of neonates produced by a *D. magna* females exposed to secondary treated effluent D (50% v/v) did not survive (Fig. 7). Primary treated effluents also significantly increased the mortality rates of *Daphnia* neonates at a concentration as low as 2.5% ($p < 0.05$). When adults were exposed to pyriproxyfen, 20% of the neonates did not survive (Fig. 8). In contrast, when exposure was continued for 2 days with the neonates, 100% mortality was observed (Fig. 8).

Body length or size of *Daphnia* also depended on the substance to which adult females had been exposed (Fig. 9 B). Furthermore, at 15 days of age, males produced following exposure to the juvenile hormone analog pyriproxyfen were significantly smaller than control females ($p < 0.05$) (Fig. 9 A).

3.3. Effluent replacement studies

No changes were observed concerning the mean broods/day (data not shown) with 3 secondary treated effluents (A, C, D) under exposure conditions in which the effluent was not renewed daily. However, the mean number of neonates/brood was significantly decreased by 40% and the mortality rate of neonates was 10% higher with effluent D (20% v/v; without renewal) compared to the control. Approximately 18% and 9% of the neonates from the third brood were males when adult female *Daphnia* were exposed to effluent C and D, respectively (20% v/v; without renewal). Both secondary treated effluents which were not renewed also caused a body size reduction (data not shown).

3.4 Ozone treatment

Only effluent 1 (50% v/v) with the lowest ozone treatment (30 mg/dm^3) elicited biological effects (Fig. 3, 5, 7, 9 B). Effluent 2 (50% v/v) at (100 mg/dm^3 ozone) and effluent 3 (50% v/v) at (300 mg/dm^3 ozone) did not caused any significant differences compared with the control (Fig. 3, 5, 7, 9 B).

4. Discussion

In the present study, diverse effects of pulp and paper effluents were observed : increased mortality, reduced fecundity, body size diminution, delayed moulting and changes of sexual phenotype. These biological effects may be associated with a variety of mechanisms including juvenile-ecdysteroid hormone interactions. Juvabione is a juvenile hormone analog

in insects (Bede *et al.,* 2000) and primary effluents were found to contain juvabione and its isoform, dehydrojuvabione (Martel *et al.*, 1997). They may also have contained other juvenile hormone analogs which may have contributed to the observed effects. Under condition of normal juvenile hormone concentration (Schema 1 A) a balance exists between juvenile hormone and ecdysteroid hormone which is necessary for normal moult, body size and sexual maturation. However, increased juvenile hormone concentration (Schema 1 B) and the associated ecdysteroid imbalance may lead to Hb2 gene induction, increased male phenotype (sex ratio), delayed moulting, growth inhibition and impaired sexual maturity. Moreover, Schema 1 B also represents the potential effects of xenobiotics which act to mimic juvenile hormone. For example, the model juvenile analog pyriproxyfen has been shown to induce the juvenile hormone receptor (JHR) reporter gene Hb2 as well as the male phenotype (Schema 1 B1, B2) (Roy *et al.*, in preparation, article I; Rider *et al.* 2005). Juvabione and primary treated effluent (10% v/v) but not secondary treated effluent were also reported to induce the expression of the gene Hb2 (Roy *et al.*, in preparation, article I).

With pyriproxyfen, the presence of male neonates agrees with other studies that found that low concentrations of Mf (EC50 27.8 µg/L) and pyriproxyfen (EC50 1 µg/L) lead to production of male *Daphnia* phenotypes in a dose-dependent manner (Rider *et al.*, 2005). The threshold concentration (0.1 µg/L) of pyriproxyfen causing the male phenotype was the same in the present study as that determined by Tatarazako and Oda (2007). However, no information concerning the reproductive capacity of the male was mentioned in previous studies. The male phenotype obtained by exposure to pyriproxyfen in the present study was unable to reproduce and was presumed to be sterile (Schema 1 B4).

Since reproduction requires several moult cycles, substances that affect moulting would also be expected to affect reproduction. Reduced brood size and delayed moulting were observed under various exposure conditions (Fig. 3 and 4; Table 3). Delayed moulting in *Daphnia* has also been reported after exposure to juvenile hormone analogs (e.g. methoprene; McKenney and Celestial, 1996) or ecdysteroid disruptors (e.g. methoxychlor; Zou and McKeown, 2005). These effects are consistent with juvenile hormone and ecdysteroid imbalances (Baldwin *et*

al., 2001; Belai and Fekete, 2003; Mu and LeBlanc, 2004; Tuberty and McKenney, 2005). The precise mechanisms of juvenile hormone analog anti-ecdysteroidal activities have yet to be elucidated (Mu and Leblanc, 2004). Such imbalances may also apply to other reproductive endpoints. For instance, nine of the ten tested chemicals known as juvenile hormone or juvenile hormone analogs, including pyriproxyfen (Tatarazako and Oda, 2007), were reported to reduce the reproductive rate in *D. magna*.

For *Daphnia* exposed to primary effluents 2.5% (v/v), effluent 1 (ozone treatment 30 mg/dm^3 at 50% v/v) and juvabione, the effects on adult correspond with the juvenoid positive control pyriproxyfen (2.6 µg/L) (Schema 1 B3). In all of these cases, the mean number of broods per day (moulting time) and the mean neonates per brood (Fig. 3 and 5) were reduced. Juvabione (2.25 µg/L) and a mixture of juvabione (1.10 µg/L) and epimanool (0.97 µg/L) exhibited the greatest effects on these two parameters in adults (Figures 3 and 5). For primary effluent, the reduction of neonates per brood was concentration-dependent (Fig. 4) as in the case of pyriproxyfen (Tatarazako and Oda, 2007). It is interesting to note possible intra-specific differences in sensitivity to juvabione. That is, the IC25 for reproductive effects in 7 day old *Ceriodaphnia dubia* was 7 µg/L (O'Connor *et al.*,1992). In contrast, 2.25 µg/L juvabione caused no *D. magna* neonate in the present study.

For *D. magna* progeny, biological effects reported in this study varied from body size diminution and changes of sexual phenotype, to severe growth arrest and early mortality (Fig. 6, 7, 8, 9). However, none of the tested substances or effluents leads to production of males (unable to reproduce sexually) observed with the positive control pyriproxyfen (Schema 1 B2). An interesting observation is the "juvenile" phenotype observed in *D. magna* exposed to all of the primary treated effluents (Fig. 6). Production of offspring with mixed larval and juvenile physical traits has also been observed with other crustaceans after exposure to Mf alone (272 to 768 µg/L) (Abdu *et al.*, 1998) or Mf plus bisphenol-A (Mu *et al.*, 2005). One possible explanation is that disruption of moulting could result in growth alterations (Laufer *et al.*, 2002).

The juvenile and male neonate phenotypes imply decreased fitness of *Daphnia* because they

were not able to reproduce. Figure 8 also illustrates that male neonates are more sensitive to the effects of pyriproxyfen than the adult females. Moreover, in the case of adults, the LC50 of primary effluent was 6 times higher than the lethal concentration for neonates (Fig. 2 and 7). These results are similar to larval versus adult sensitivity to juvenile hormone analogs in other crustaceans such as the grass shrimp (*Palaemonetes pugio*) and mud crab (*Rhithropanopeus harrisii*) (Tuberty and McKenney, 2005). Standard bioassays with *D. magna* rely primarily upon adult mortality and gross indicators of reproduction (OECD, 2006). However differences in adult/neonate sensitivity suggest that endpoints should include several parameters relating to the neonates.

Balsam fir, a prominent species used in thermomechanical pulp mills in Québec, contains juvabione and dehydrojuvabione and extracts of this tree were found to be particularly toxic to the crustacean *Ceriodaphnia dubia* under chronic conditions (O'Connor *et al.*, 1992). A series of primary effluent samples, including those tested in the present study, from the same thermomechanical mill was found to contain relatively high concentrations of juvabione and dehydrojuvabione (Table 2). The mean juvabione concentration was estimated to be 9.5 mg/L which is consistent with other primary effluents (Martel *et al.*, 1997). Juvabione inhibited embryo development in insects (Zaoral and Slama, 1970) and egg hatching in spruce budworms (*Choristoneura fumiferana*) (Retnakaran, 1970). The EC25 for Ceriodaphnia reproduction was reported to be 0.5 µg/L for dehydrojuvabione (O'Connor *et al.*, 1992). In the present study, the relatively low concentration of juvabione (2.25 µg/L) inhibited the production of neonate (Fig. 3).

In contrast to primary effluents, the juvabione and dehydrojuvabione concentrations were reduced by more than 99% in secondary effluents (Table 2). Similarly, secondary treatment of pulp mill effluents decreased juvabione and dehydrojuvabione by approximately 90% (Martel *et al.*, 1997). In the present study, secondary effluents were found to be generally less toxic and demonstrated a qualitatively different battery of biological effects (Table 3, Fig. 6 and 7). Secondary treated effluent D is an exception, but RFA and juvabione concentration were not analysed in this effluent.

As shown previously, the present study reported that reduction in body length of crustacean neonates was correlated with reduced fecundity (Nimmo *et al.*, 1981; Verslycke *et al.*, 2004 from OECD, 2006) and delay moulting in adults (Table 3, Schema 1 B3 and B4). However, production of a phenotype "juvenile" (Fig. 1 C) and the fact that males are smaller than females (Fig. 9 A) could be confounding factors for body size analysis. In fact, substances causing the "juvenile" phenotype (Fig. 6) were expected to cause a body size reduction in 8 day-old *D.magna* (Fig. 9 B). Moreover, the body length selected for "juvenile" corresponded approximately to the size of 2 day old normal female *D. magna* (around 1.6 mm) (Johnson and Delaney, 1998). Because of those confounding factors, decrease in body length did not seem to be a good bioindicator to be combined with changes in phenotype (juvenile and male).

Effluent exposures were conducted under several different conditions. A moderate effect was associated with maintaining the same effluent for period of 6 days or renewing the effluent daily (data not shown). In general, notable differences occurred between effluents compared to relatively minor differences for effluent renewal (Table 3). However, this information confirmed that bioindicators tested could be useful for comparison between secondary treated effluents. It is suggested that ozone treatment at concentrations of 100 mg/dm^3 ozone or above effectively diminished effluent toxicity (Fig. 3, 5, 7, 9) as reported in Pokhrel and Viraraghavan (2004).

In summary, the results of exposure to juvabione and primary mill effluents are consistent with mechanisms involving juvenile-ecdysteroid hormone interactions. Qualitatively different sexual phenotypes were produced : male neonates (unable to reproduce sexually) and juvenile neonates. It is recommended that bioassay endpoints include delayed moulting and changes in neonate phenotypes.

Acknowledgments

We thank Valerie Nash for supplying *Daphnia magna* for our experiments as well as technicians of PAPRICAN for chemicals analysis of effluents. This work was supported by an FQRNT scholarship awarded to M-E R.

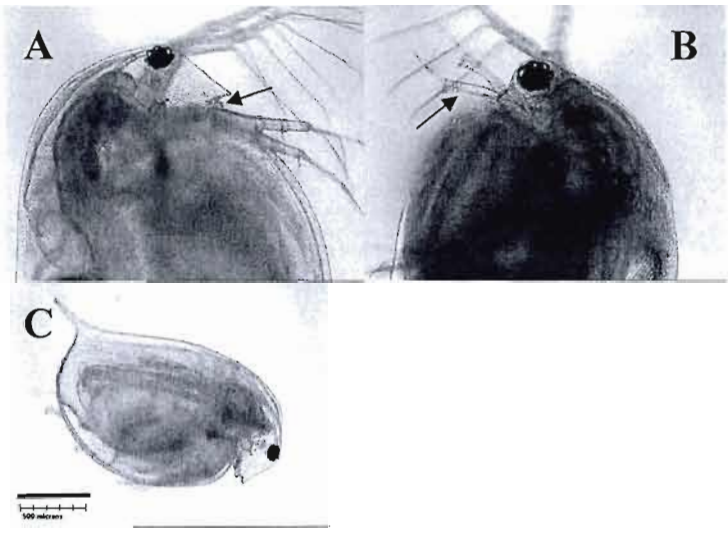


Figure 1. Picture of 9 days old *Daphnia* grown in clear water. (A) Female from control, (B) male from pyriproxyfen and (C) juvenile from primary treated effluent. Arrows represent the first antenna in the female and the male. For all pictures, the scale is a black line that represents 500 µm

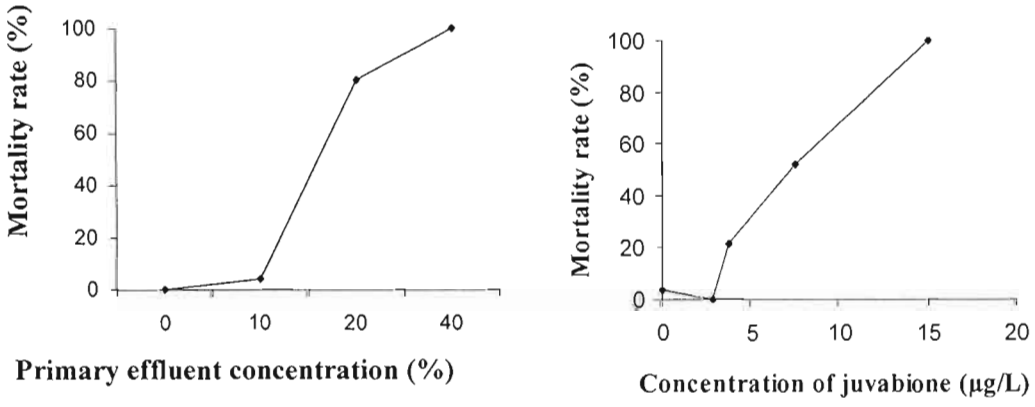


Figure 2. Mortality rate of *D. magna* exposed during 48 h to different concentrations of one primary effluent and juvabione. For all the concentrations of primary effluent n = 25. For juvabione, at 0 µg/L (n = 28), at 2.85 µg/L (n = 22), at 3.75 µg/L and 7.5 µg/L (n = 27) and at 15 µg/L (n = 28).

Note. At concentration of juvabione above 3.75 µg/L, all the neonates born during the exposition were dead

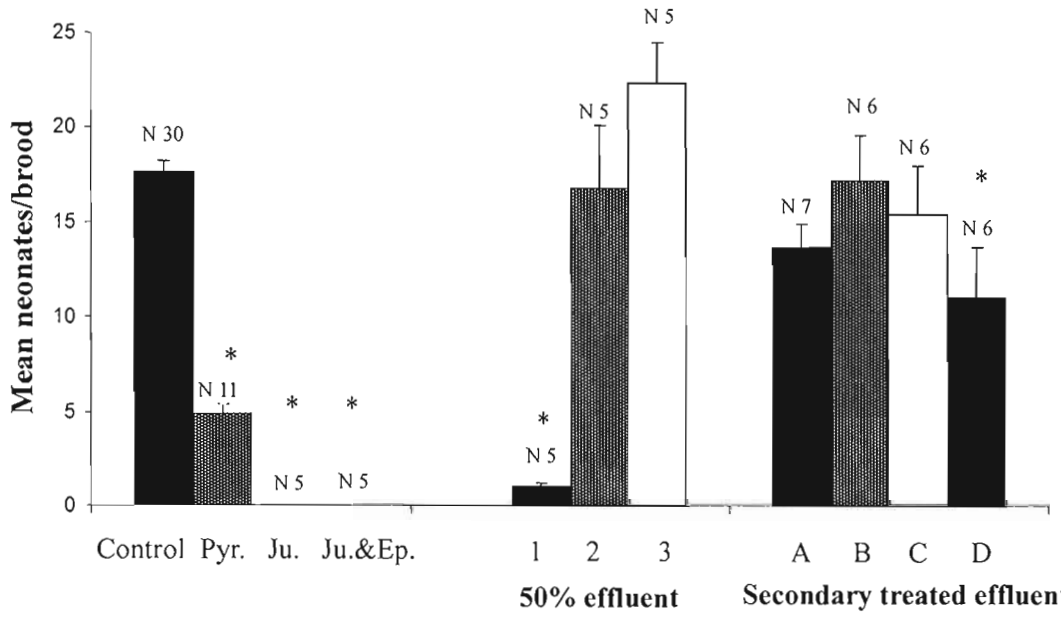


Figure 3. Mean number of neonates per brood for *D. magna* exposed to pure chemicals (Pyr. = pyriproxyfen (2.6µg/L)), or natural substances (Ju. = juvabione (2.25 µg/L), Ju. and Ep. = juvabione (1.1 µg/L) and epimanool (0.97 µg/L)) or effluents (A = 40% v/v, B, C and D = 50% v/v). Letter A, B, C and D represent different effluents. Effluent 1(30 mg/dm^{3}ozone), 2 (100 mg/dm^{3}ozone), 3 (300 mg/dm^{3}ozone). Bars represent standard errors. Asterisk indicates statistically significant difference between treated and control group

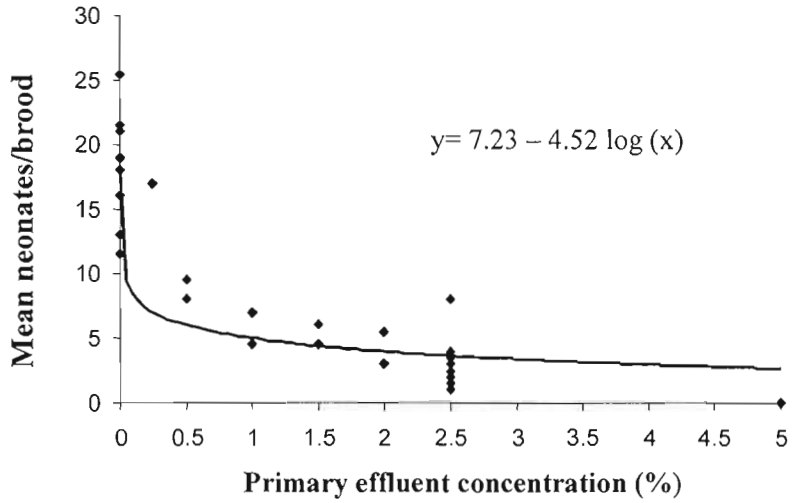


Figure 4. Mean number of neonates per brood for *D. magna* exposed to primary treated effluents. The best curve fitting model has a $R^2 = 0.81$, $p < 0.0001$ and n = 35

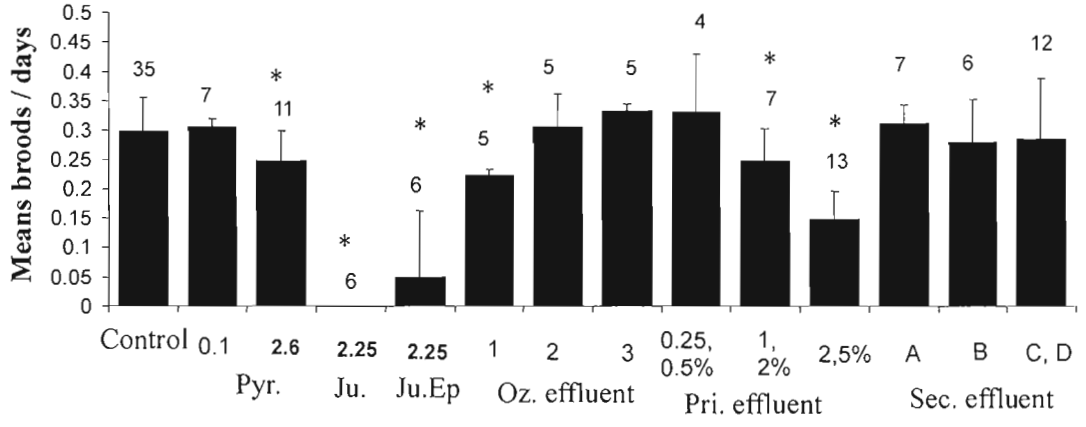


Figure 5. Mean number of brood per day by *D. magna* expose to different substances (Pyr = pyriproxyfen, Ju. = juvabione, Ju.Ep. = 49% juvabione and 43% epimanool) with concentrations in µg/L. All effluent concentrations are in % (v/v). Oz. effluent = ozone effluents at 50% : 1 (30 mg/dm^3 ozone), 2 (100 mg/dm^3 ozone), 3 (300 mg/dm^3 ozone). Sec. effluent = secondary effluents (A = 40% v/v, B, C and D = 50%). Pri. Effluent = primary effluents. Differences between control and treatment were tested by Wilcoxon test. Bars represent standard errors. Numbers above the bars represent different n. Asterisk indicates statistically significant difference between treated and control group

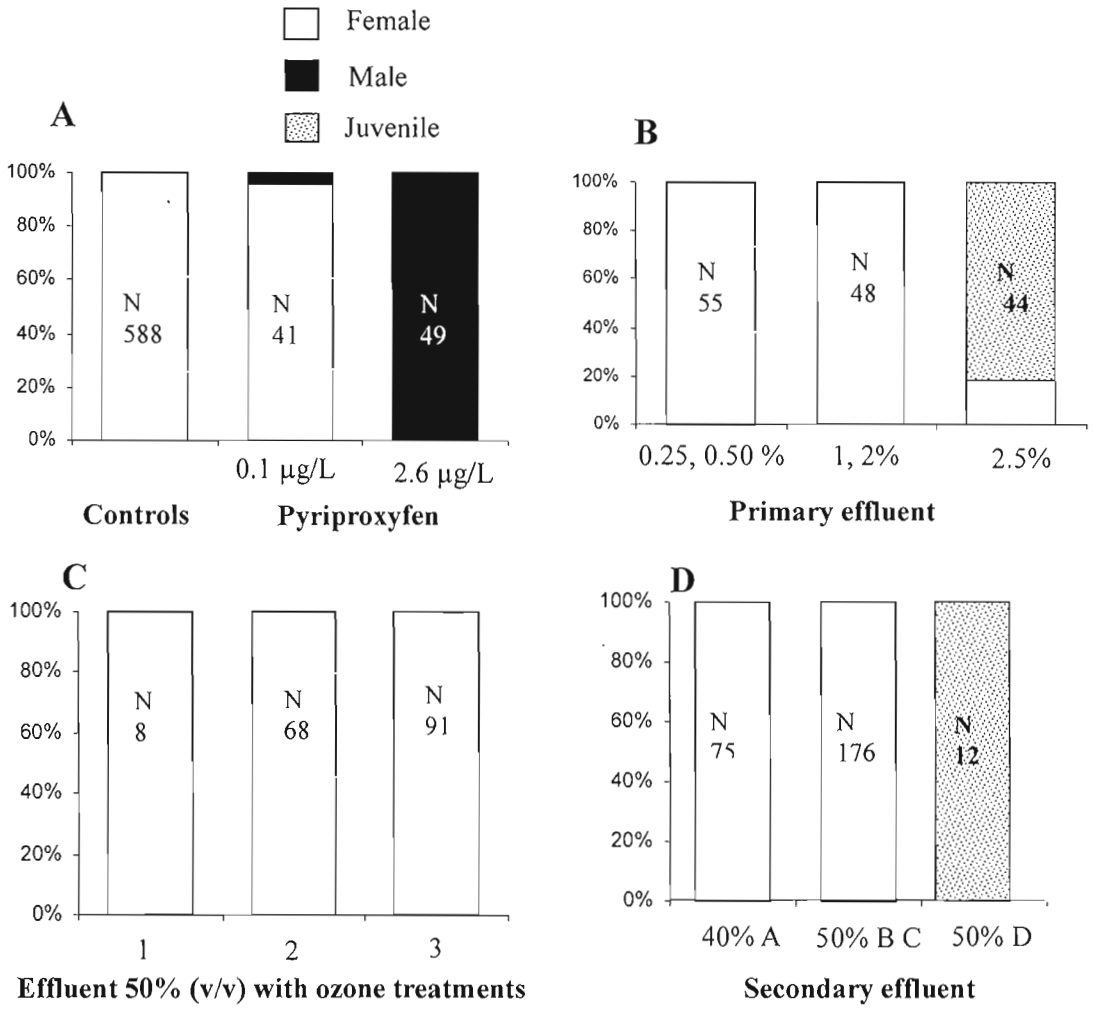


Figure 6. Percentage of sexual phenotype of the third brood of *D. magna* with prenatal exposure to (A) pyriproxyfen, (B) primary effluents, (C) 50% effluent 1(30 mg/dL^3), 2 (100 mg/dL^3), 3 (300 mg/dL^3) and (D) secondary effluents. For each treatment the number of replicas varies between 3 and 36 dues to mortality.

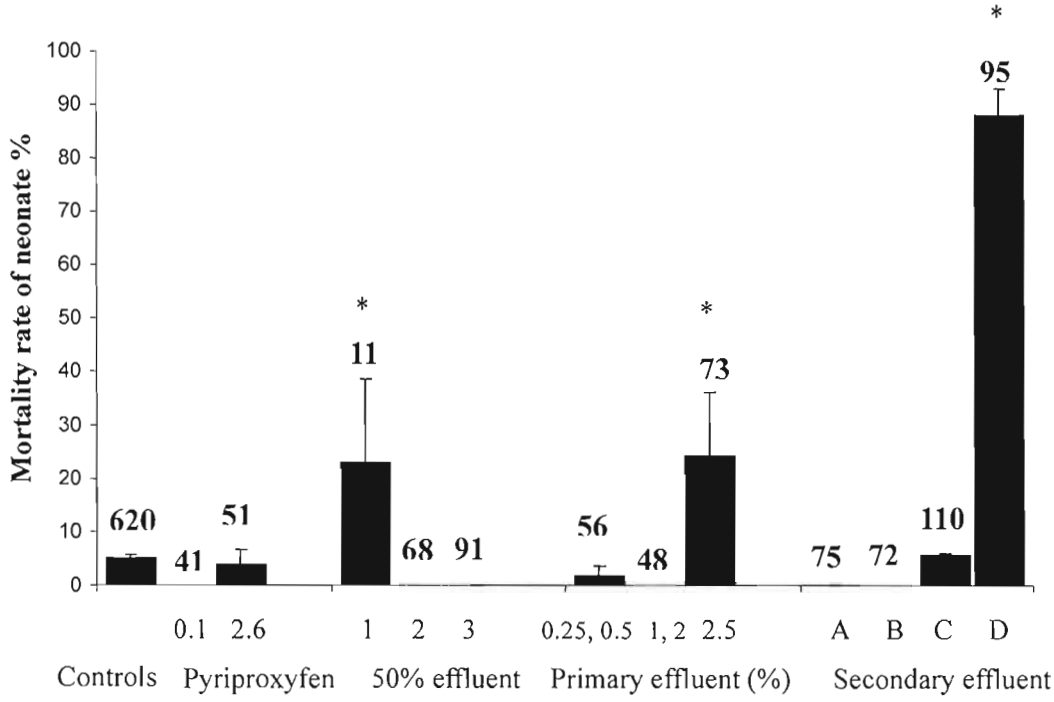


Figure 7. Mortality rate : percentage of neonates *D. magna* from the third brood with prenatal exposition to pyriproxyfen (concentration in µg/L) or to different effluents (concentrations in % v/v) that did not survive during the 8 first days. Concentrations of secondary treated effluents : A = 40%, B, C, D = 50%, effluent 1 (30 mg/dm^{3}ozone), 2 (100 mg/dm^{3}ozone), 3 (300 mg/dm^{3}ozone) = 50%. Bars represent standard errors. Numbers above the bars represent different n. Asterisk indicates statistically significant difference between treated and control group

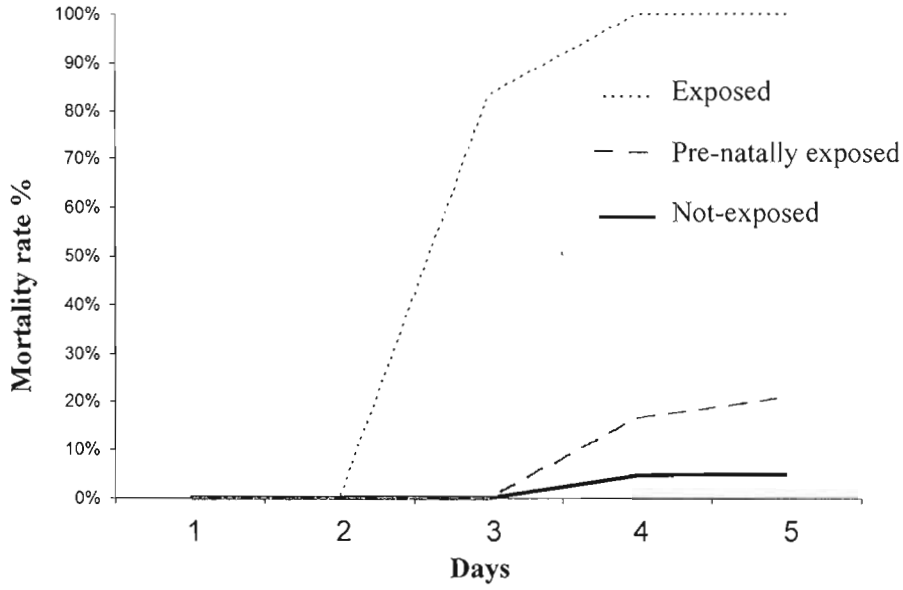


Figure 8. Mortality rate in % of neonates in culture water from *Daphnia* not exposed to pyriproxyfen (n = 21), compared to neonates in culture water from *Daphnia* exposed to pyriproxyfen 2.6 µg/L (n = 18), as well as neonates with 2 days exposition to pyriproxyfen 2.6 µg/L also from *Daphnia* exposed to pyriproxyfen (n = 18)

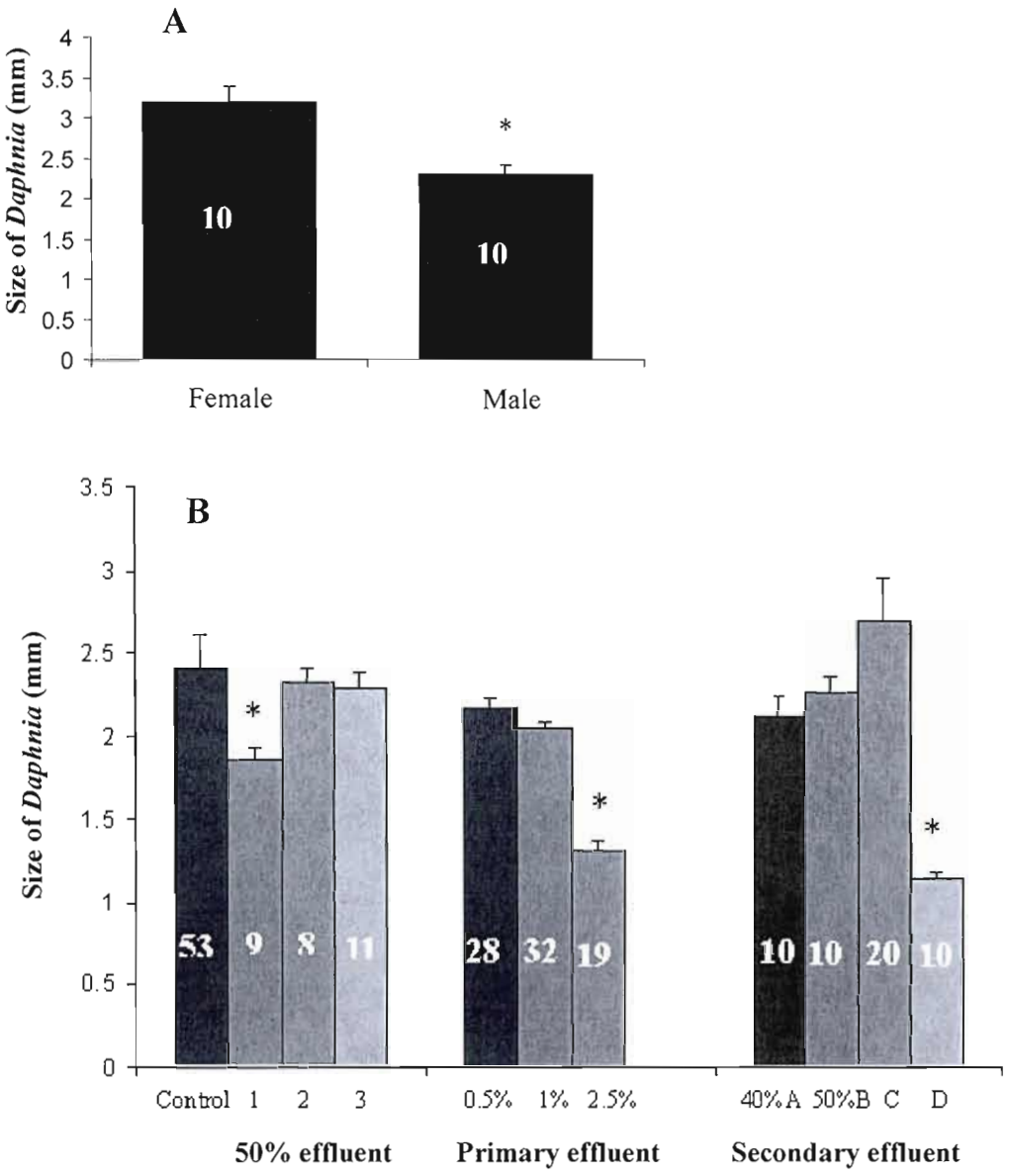
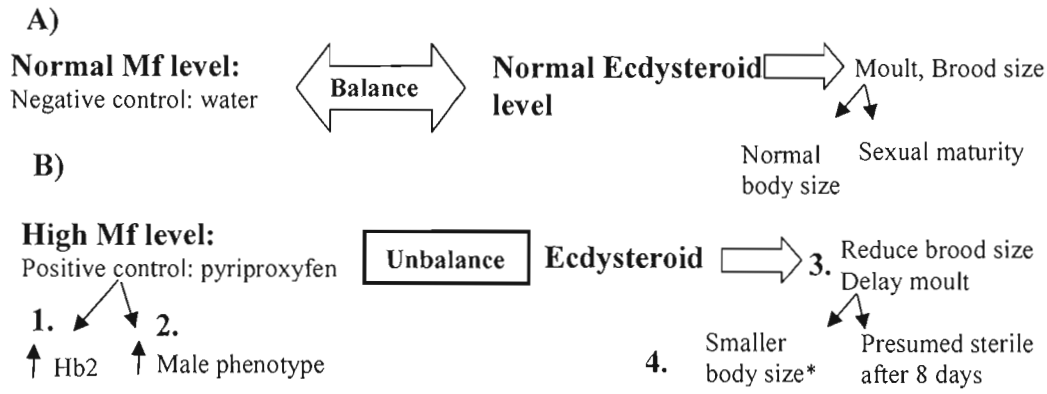


Figure 9. (A) Body size of 15 days old females and males *D. magna* kept in culture water. Female come from control and male from pre-natal exposure to pyriproxyfen (2.6 µg/L). (B) Body size of 8 days old *D. magna* with pre-natal exposure to different effluents kept in culture water. Effluent concentrations are in % (v/v). Effluent A = 40% and effluent B, C, D = 50 %. The body size was measured from the point immediately above the eyespot to the base of the dorsal spine. Standard errors are represented by the bars. Numbers in the columns represent different n. The asterisk above the bars indicates statistically significant difference between body size of *Daphnia* exposed to different substances compared to the control



Schema 1. Model of normal and excess level of the juvenile hormone methyl farnesoate (Mf) in *D. magna* link to ecdysteroid. A) Balance between normal level of Mf and ecdysteroids are represented by exposure to the negative control. B) Juvenoid effects (increase of Mf) are represented by the positive control and correlated with (B1) induction of the gene Hb2, (B2) increase male phenotype and inhibition of the balance with normal ecdysteroid level. Disruption of ecdysteroid balance can affect (B3) moult and growth on adult *Daphnia* as well as (B4) sexual maturation and size on their progeny. Arrows pointing upward represent an increase; other arrows are used to represent interactions. Asterisk indicate that male phenotype from pyriproxyfen had smaller body size than female from negative control

Table 1. Basic water and maintenance conditions for *D. magna.*

Parameters	Frequency	Measure
Temperature	Constant	20-22°C
pH	Weekly	7±2
Dissolved oxygen	Weekly	6-8 mg/l
Conductivity	Weekly	1050-1200 µmhos
Light intensity	Weekly	400-800 lux
Photoperiod day/night		16L : 8D
Water source		30% distilled water and 70% filtered well water
Diet composition		Yeast cerophyll-trout chow YCT and *Selenastrum capricornutum*
Regimen - 1L	Daily	5 ml *Selenastrum* for 2 ml YCT
Regimen - 40 ml	Daily	0.4 ml *Selenastrum* for 0.2 ml YCT

Table 2. Chemical analysis for RFA (resin and fatty acid), BOD (biochemical oxygen demand), juvabione and dehydrojuvabione in effluents. Same letter represent same effluent. *The asterisk indicates effluent not tested in this study, but obtained from the same mill with the same treatment plants and the same sampling methods that the one used in this study. D = detected, ND = not detected (detection limit 10 µg/L).

Effluent	Treatments	RFA (mg/L)	BOD (mg/L)	Juvabione (mg/L)	Dehydro Juvabione (mg/L)
A	Secondary	0.23	23	0.02	0.02
B	Primary				
B	Secondary	0.02		ND	ND
C	Secondary		8		
C1	Ozone 30		13		
C2	Ozone 100		24		
C3	Ozone 300		32		
D	Primary				
D	Secondary				
E	Primary			D	D
***F**	Primary	11.4		6.44	1.06
***F**	Secondary	0.05		0.04	ND
***G**	Primary	25.4		8.9	5.9
***G**	Secondary	0.1		ND	ND
***H**	Primary	19.84		13.15	3.98
***H**	Secondary	0.08		0.02	0.01

Table 3. Summary table of three physiological indicators in *D. magna* : moulting time, brood size and body size. Arrows pointing down mean a significantly decrease compared with control. N.A. means not applicable.

Substances	Concentration µg/L or %(v/v)	Moulting time Mean brood/days	Brood size	Body size
Pyriproxyfen	2.6 µg/L	↓	↓	↓
Juvabione	2.25 µg/L	↓	↓	N.A.
Juvabione / epimanool	2.25 µg/L	↓	↓	N.A.
Primary effluent	2.5%(v/v)	↓	↓	↓
Secondary effluent renew (except D)	50%(v/v)	—	—	—
Effluent D renew	50%(v/v)	—	↓	↓
Secondary effluent not-renew A	40%(v/v)	—	—	—
Effluent D not-renew	20%(v/v)	—	↓	↓

CONCLUSION

1. Utilisation d'un biomarqueur génétique et d'indicateurs biologiques pour la détection de pollutions juvénoïdes

Certains produits botaniques et pesticides agricoles synthétiques sont une source de substances juvénoïdes (Bede, 2000; Tatarazako et Oda, 2007). Cependant, les substances ayant des activités juvénoïdes peuvent se retrouver dans les écosystèmes, notamment dans les milieux aquatiques et affecter des organismes non- ciblés (Bertold, 2005; Lafront, 2000; Leach *et al.*, 1975). Les invertébrés aquatiques chez qui l'hormone juvénile Mf a été repérée notamment de nombreuses espèces de crustacés (Tobe et Bendena, 1999), peuvent ainsi être affectés par ces substances. Le biomarqueur génétique utilisant l'augmentation du gène Hb2 testés dans cette étude a permis la détection de substances juvénoïdes notamment les effluents primaires et la juvabione. Il devient ainsi possible de détecter et d'évaluer les effets juvénoïdes de d'autres substances naturelles pouvant se retrouver en milieux aquatiques (ex. déhydrojuvabione, juvabiol et plusieurs composantes d'huiles végétales; Manville, 1976) chez les crustacés, en utilisant le biomarqueur et les indicateurs biologiques chez *D. magna*. L'identification de substances ou de mélanges présents dans l'eau, avec des effets juvénoïdes, est une étape importante afin de diminuer leur propagation en milieux non- désirables. De plus, les résultats présentés dans cette recherche indiquent que le biomarqueur et les indicateurs biologiques pourraient potentiellement servir à identifier des substances avec des activités anti-ecdystéroïdes.

Les perturbateurs endocriniens sont des exemples de l'influence de la pollution environnementale sur le phénotype des êtres vivants. La détection de substances ayant des activités juvénoïdes (article I) et le test avec des indicateurs biologiques (article II) peuvent permettre de faire des liens entre le système de signalement hormonal et les effets sur les organismes. Par exemple, dans cette étude, l'analogue de l'hormone juvénile (i.e. pyriproxyfen) est lié à la production de mâles qui ne se reproduise pas. De plus, des

substances détectées juvénoïdes comme les effluents primaires et la juvabione sont liées respectivement avec la présence du phénotype juvénile et l'inhibition de la ponte.

Les hormones rétinoïques ont une structure semblable aux juvénoïdes des invertébrés aquatiques (Tobe et Bendena, 1999). L'hormone juvénile ainsi que certaines substances connues avec des activités d'hormones juvéniles (ex. méthoprène et certains métabolites) sont capables d'imiter l'action des rétinoïdes (ex. l'acide trans-rétinoïque) en agissant sur le récepteur rétinoïde X (RXR) et vice-versa (Gade *et al.*, 1997; Harmon *et al.*, 1995; Wang *et al.*, 2005). Il serait intéressant d'appliquer notre test (biomarqueur génétique et indicateur biologique avec *D. magna*) afin de tester des substances ayant des activités rétinoïques (ex. acide oléique).

De plus, les effluents d'usines de pâte et papier thermomécaniques pour la fabrication du papier journal sont connues pour leurs activités rétinoïques en se liant au récepteur rétinoïque RXR chez certains poissons (Alsop *et al.*, 2003). Il est difficile de faire des rapprochements entre les effets endocriniens chez les arthropodes comme la *Daphnia magna* et ceux des vertébrés aquatiques, notamment les poissons. Cependant, il est possible que les substances juvénoïdes (ex. juvabione) affectent aussi le système rétinoïque des vertébrés.

2. Apport de cette étude pour les recherches environnementales sur les effluents d'usines de pâte et papier thermomécaniques

L'induction du gène Hb2 a permis de détecter la juvabione et les effluents primaires comme étant des substances juvénoïdes chez les crustacés. Ces informations sur le mécanisme d'action des effluents et de la juvabione peuvent servir à comprendre les effets observés dans des recherches futures. Des substances affectant le système des ecdystéroïdes (ex. anti-ecdystéroïdes) semblent être présentes dans certains effluents d'usines de pâte et papier (Brynda *et al.*, 2001). Comme nous avons vu dans l'article II, il est possible que les juvénoïdes présents dans les effluents soient en partie responsables de ces effets.

Actuellement dans la recherche environnementale sur les effluents d'usines de pâte et papier, peu de test avec les crustacés ne détectent les modulateurs juvénoïdes ou ecdystéroïdes. L'étude sur les indicateurs biologiques avec *D. magna* (article II) pourrait donc combler ce besoin. La méthodologie développée est simple, peu dispendieuse et permet de détecter des effets sur le phénotype à faible concentration.

Les résultats présentés montrent que le temps de la mue et la diminution du nombre moyen de néonates par portées, chez la daphnie, varient avec le traitement des effluents. En générale avec le traitement secondaire, qui enlève une grande proportion de juvabione, la plupart des effets d'anti-ecdystéroïdes présents dans les effluents primaires semblent être éliminés. De plus, les résultats permettent de constater que des tests sur des daphnies matures seulement ne sont pas aussi efficaces pour observer des effets juvénoïdes et anti-ecdystéroïdes que des tests qui incluent les effets sur leurs progénitures. En effet, dans notre étude, il fut possible d'observer un nouveau phénotype chez les néonates : les juvéniles. L'exposition aux effluents peut aussi affecter la survie des néonates sans toutefois être détecté chez l'adulte. En plus, avec les effets sur les néonates, ce fut possible d'observer des différences entre différents effluents secondaires.

Plusieurs facteurs peuvent expliquer la différence de toxicité entre les effluents secondaires, comme le pH (Pranovich *et al.*, 2003), la concentration d'acides gras et de résines (RFA) (McLeavy, 1987; Qin et al., 2004) et la demande biochimique en oxygène (BOD) (Kantardjieff & Jones, 1997). Les concentrations de RFA dans les effluents secondaires sont évaluées entre 0.05 et 0.35 mg/L (McLeavy, 1987) et un BOD de moins de 35 mg/L est considéré comme non-toxique pour *D. magna* (Kantardjieff & Jones, 1997). Dans notre étude, les effluents secondaires analysés pour le niveau de BOD et de RFA n'ont pas permis d'expliquer la différence de toxicité entre ceux-ci.

3. Les propriétés des substances naturelles des plantes

Le terme modulateur endocrinien englobe des milliers de molécules synthétique (Defur, 2004). Cependant, dans cette étude, ce ne sont pas seulement des produits synthétiques, mais aussi des effluents et une substance naturelle qui sont testés comme modulateur. Les substances naturelles des plantes peuvent être bénéfiques pour les écosystèmes forestiers. Par exemple, elles permettent à certains arbres de se défendre eux-mêmes contre des prédateurs vu leurs effets stérilisant sur plusieurs espèces d'insectes en bloquant le début de la mitose (Salehzadeh *et al.*,2003). Certains terpénoïdes comme la juvabione sont aussi reconnus pour avoir des propriétés antibactériennes et fongicides, en empêchent complètement leur croissance, afin de protéger le bois (Aoyama *et al.*, 1991; Lindberg et Holmbom, 2004). Aussi, des inventions et des brevets pour synthétiser des juvénoïdes comme la juvabione et ses dérivés sont faits dans l'intention d'utiliser ces substances comme insecticides ou fongicides (ex. juvabione synthétisé à partir du 4-(2-Formyléthyle) cyclohexanone) (Itagaki et Iwabuchi, 2007).

Notre étude démontre que la juvabione est une substance juvénoïde avec des effets anti-ecdystéroïdes (inhibition de la mue et de la ponte) chez les crustacés notamment *D. magna*. En améliorant les connaissances sur les propriétés de la juvabione, il fut possible dans cette étude de cibler cette substance comme potentiellement responsable d'une partie des effets observés dans les effluents primaires.

4. Limite de cette étude et possibilités futures

La méthode de détection du changement d'expression du gène Hb2 peut se faire après une exposition de 24 heures seulement, mais nécessite plusieurs étapes en laboratoire. Néanmoins, après quelques jours, un changement de couleur chez les *D. magna* (parfois de beige à rouge foncé) était facilement observable. Peut-être que ce changement de couleur pourrait être corrélé avec l'augmentation du gène de l'hémoglobine Hb2. Deuxièmement, les

niveaux d'augmentation du gène Hb2 par l'hypoxie pourraient être quantifiés avec plus de données. Je suggère donc dans une étude future de regarder comment l'induction du gène Hb2 varie en fonction de la concentration d'oxygène. Ainsi, il serait peut-être plus facile de déterminer si une augmentation du gène Hb2 est induite par une substance juvénoïde ou par l'hypoxie.

Le rapprochement entre les concentrations de substances pures et leurs effets en solution est difficile à faire. Vu cette limite, il ne fut pas possible de faire des comparaisons directes entre la concentration de juvabione dans les effluents et la juvabione testée comme substance pure. De plus, comme la juvabione est une substance repérée comme ayant une biodégradation rapide (O'Connor *et al.*, 1992; Martel *et al.*, 1997), une exposition à flot constant pourrait aider à maintenir la concentration de juvabione pendant de plus longue période de temps.

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