An investigation of pulp mill effluents and their wood feedstocks as potential neuroendocrine disruptors of the fish reproductive axis

Andrew Waye

A thesis submitted to

The Faculty of Graduate and Postdoctoral Studies

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biology

University of Ottawa

Ottawa, ON, Canada

Abstract

Common observations of reduced gonad size and spawning inhibition in wild and laboratory raised fish exposed to pulp mill effluents indicate that reproductive neuroendocrine signalling pathways may be upset. This thesis supported the neuroendocrine disruption of reproduction hypothesis by identifying potential disruptors and targets where these impacts may occur. A mechanistic study of the *in vivo* fathead minnow (FHM) spawning assay used by industry to assess effluent quality showed that ovulation, but not milt production, was impaired. This finding supported the hypothesis that the neuroendocrine cascade that triggers ovulation may be disrupted. I hypothesized that neuroactive constituents previously described in effluents were originating in wood feedstocks and neuroactive extracts of hardwood and conifer feedstocks were identified. Phytochemicals associated with effluents were neuroactive. Structurally similar phenolic phytochemicals showed monoamine oxidase (MAO) inhibition, and resin acid diterpenes displayed glutamic acid decarboxylase (GAD) inhibition. Inhibitors of these enzymes may have impacts on the control of reproduction since MAO metabolizes dopamine, an inhibitor of the neuroendocrine reproductive axis, while GAD synthesizes γ-aminobutyric acid (GABA), a stimulator of this axis. Bioassay-guided fractionations of effluents and wood feedstocks identified that medium polar extracts of primary- and secondary-treated effluents and balsam fir feedstock contained high GAD inhibitory activity. This activity was associated with chemically complex fractions rather than single active principles. Advanced metabolomic comparison of medium polar extracts of feedstock and treated effluent identified 15 common plant metabolites, demonstrating that phytochemicals entering the mill in wood are surviving pulp production and effluent treatment processes and may be responsible for observed GAD inhibition. Discriminant metabolomics analysis identified 4-acetylpyridine, a novel compound to be described in effluents, as well as two other tentatively identified compounds, as chemical markers of GAD inhibitory effluent fractions. Five tentatively identified chemical markers and (+)-lariciresinol were found in inhibitory balsam fir feedstock fractions. Neuroendocrine pathways that control reproduction in fish, such as dopamine and GABA pathways, are also important drug targets for the treatment of neurological disorders in mammals; therefore these results also have implications for the development of natural health products from phytochemicals and tree extracts common to Canadian forests. By using an interdisciplinary approach (phytochemistry, neuroendocrinology, ecotoxicology), I was able to explore the various implications of my research on the fields of natural health products chemistry and aquatic toxicology.

Résumé

Une réduction de la taille des gonades et l'inhibition de la fraie, observées chez les poissons d'élevage en laboratoire et en milieu naturel, et qui ont été exposés aux effluents des usines de pâte à papier, indiquent que les voies de signalisation neuroendocrine de la reproduction peuvent être perturbées. La recherche présentée dans cette thèse supportait l'hypothèse de la perturbation neuroendocrine de la reproduction en identifiant des perturbateurs potentiels et des cibles où ces impacts peuvent se produire. Une étude méchanistique de l'expérience sur la fraie du Pimephales promelas *in vivo*, utilisée par l'industrie afin d'évaluer la qualité de ses eaux usées, a démontré que l'ovulation, mais pas la production de laite, était affaiblie. Ce résultat supportait l'hypothèse que la cascade neuroendocrine qui déclenche l'ovulation pourrait être perturbée. J'ai émis l'hypothèse que les constituants neuroactifs précédemment décrits dans les effluents provenaient des matières premières de bois et des extraits neuroactifs de bois franc et de conifères ont été identifiés. Les composés phytochimiques associés aux effluents étaient neuroactifs, spécifiquement, des composés phytochimiques phénoliques structurellement similaires ont montré de l'inhibition de la monoamine-oxydase (MAO), et des acides diterpènes de résine ont démontré de l'inhibition d'acide glutamique décarboxylase (GAD). Les inhibiteurs de ces enzymes peuvent avoir des impacts sur le contrôle de la reproduction puisque la MAO métabolise la dopamine, un inhibiteur de l'axe neuroendocrinien de la reproduction, alors que le GAD synthétise l'acide gamma-amino butyrique (GABA), un stimulateur de cet axe. Des fractionnements, guidés par le bioessai, des effluents et des matières premières de bois ont permis d'identifier que les extraits polaires moyens d'effluents de phases de traitements primaire et secondaire et de sapin baumier contenaient une activité élevée d'inhibition de GAD. Cette activité était associée à des fractions chimiquement complexes plutôt que de principes simples et actifs. Une comparaison métabolomique avancée des extraits polaires moyens de matières premières de bois et des eaux usées traitées a permis d'identifier 15 métabolites communs, démontrant que les composés phytochimiques intrants du moulin à bois survivent à la production de la pâte et aux processus de traitement des effluents et pourraient être responsable de l'inhibition de GAD observée. Une analyse métabolomique discriminante a identifié 4-acetylpyridine, un composé nouveau à décrire dans les effluents, de même que deux autres composés identifiés à titre d'essai, en tant que marqueurs chimiques de fractions d'effluents d'inhibition de GAD. Cinq marqueurs chimiques identifiés à titre d'essai et (+)-lariciresinol ont été trouvés dans les fractions d'inhibition de sapin baumier. Les voies neuroendocrines qui contrôlent la reproduction chez le poisson, tels la dopamine et GABA, sont aussi des cibles importantes de produits pharmaceutiques pour le traitement de désordres neurologiques chez les mammifères ; par conséquent, ces résultats comportent également des implications pour le développement de produits de santé naturelle à partir de composés phytochimiques et d'extraits d'arbres qui sont abondants dans les forêts canadiennes.

Acknowledgements

I dedicate this work to my parents. Without their support and belief in everything I do, I would not be where I am today. I would also like to thank my friends and family, and especially A. Saldanha, for support, patience, and understanding while completing this thesis.

Without the experts on whom I could draw a wealth of information and experience, this thesis would not be possible. I am forever indebted to Drs A. Saleem and J. A. Guerrero-Analco, who were patient and capable mentors, for their invaluable training in chemistry, their time, and their help in achieving my research goals.

I would like to express my gratitude for the productive research collaborations established during this thesis. The support provided by FPInnovations was invaluable to the success of this project. Thanks go to Drs T. Kovacs and B. O'Connor for the use of facilities and other resources, and particular thanks to Dr. P. Martel and his team of aquatic technicians whose help and guidance were pivotal in the success of my research on fathead minnows. At Environment Canada, special thanks to Drs. L. M. Hewitt and C. B. Milestone for providing samples and including me as a partner in their research program on pulp mill effluents.

I have heartfelt appreciation for the past and present members of the Trudeau, Arnason, Durst, and Foster labs for their support and input, with special thanks going to past lab-members Dr. W. Lado, G. Picard, M. Annal, A. Tang, C. Sun, Dr. N. Basu, R. Liu, M. Taylor, J. Mao, and B. Harnois as collaborators who assisted in generating the samples, data, and analyses presented in this thesis. I am also thankful to Dr. J. Basu and the members of my thesis advisory committees, Drs. F. Pick, B. Foster, C. Charest, and J. Blais for their advice and time, and to C. Bédard for translation of the abstract.

Support of this project was funded by NSERC and FPInnovations to the Trudeau and Arnason labs, and I am personally grateful for funding supplied by the NSERC postgraduate scholarship.

Lastly, and most importantly, I would like to acknowledge the support and guidance of my supervisors, Drs V. L. Trudeau and J. T. Arnason, who were never further than a knock on the door or a phone call away, and for that I am eternally grateful. Without them and their dedication to their students, the success of this research would not have been possible.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
D1	Dopamine type 1 receptor
D2	Dopamine type 2 receptor
DA	Dopamine
DOPAC	3,4-Dihydroxyphenylacetic acid
FHM	Fathead minnow
FSH	Follicle-stimulating hormone
GABA	g-Aminobutyric acid
GABA(A)	GABA type A receptor
GABA(A)-BZD	GABA(A) benzodiazepine-binding site
GABA-T	GABA transaminase
GAD	Glutamic acid decarboxylase
Glu	Glutamic acid
GnRH	Gonadotropin-releasing hormone
GSI	Gonadosomatic index
K	Condition factor
LH	Luteinizing hormone
mAChR	Muscarinic acetylcholine receptor
MAO	Monoamine oxidase
NMDA	N-methyl-D-aspartate receptor
RO	Reverse osmosis
SSA	Succinic semialdehyde
UPLC-QTOF	Ultra-high performance liquid chromatography – time of flight mass spectrometry

Chapter 1: General introduction

1.1 Introduction to thesis

Reproductive impairment is often observed in fish exposed to pulp mill effluents, and has been a major environmental problem in Canada and other pulp and paper producing countries. Across Canada, the Environmental Effects Monitoring program identified a national trend of reduced gonad size in wild fish exposed to effluent (McMaster et al 2006), while in controlled laboratory effluent quality monitoring, spawning is rapidly inhibited in fathead minnows (*Pimephales promelas*) (Kovacs et al 2007, 2013). Complex interactions of the effluents, species variability of both fish and pulp tree feedstocks, and multiple biological pathways have complicated research efforts over the last 20 years to identify the biological mechanism(s) and active principles(s) responsible for these reproductive effects (Barrett et al 2010, Hewitt et al 2006, McMaster et al 2006, Parrott et al 2006). While studies have demonstrated steroid-dependant pathways at the levels of the gonad and liver are clearly affected, modulation of other fish signalling pathways by pulp effluents may be involved (Basu et al 2009, Van Der Kraak et al 1992, Waye & Trudeau 2011) since many anti-reproductive effluents are neither estrogenic or androgenic in standard assays (Hewitt et al 2006, McMaster et al 2006, Parrott et al 2006). This thesis explores a novel mechanism by which effluents are inhibiting reproduction in fish: neuroendocrine disruption caused by pulp mill effluents and their feedstocks. Each chapter of this thesis is written as a stand-alone manuscript to be published as a research article in a peer-reviewed journal.

1.2 Thesis Rational

1.2.1 The neuroendocrine disruption hypothesis

In this thesis, I proposed that the anti-reproductive effects of effluents observed in wild and lab fish studies could result in part from neuroendocrine disruption of the hypothalamus-pituitary-gonad reproductive axis. This hormonal axis is present in all vertebrates and in the following research chapters, I identified neuroendocrine pathways in fish that could be disrupted by exposure to pulp and paper mill effluents. I adopted the definition of neuroendocrine disruptors as: “pollutants in the environment that are capable of acting as agonists/antagonists or modulators of the synthesis and/or metabolism of

neuropeptides, neurotransmitters, or neurohormones which can subsequently alter diverse physiological, behavioural, or hormonal processes to affect an animal's capacity to reproduce, develop and grow, or deal with stress and other challenges (Waye & Trudeau 2011)". I provided a comprehensive and broadly focussed published review (Waye & Trudeau 2011) of the literature on neuroendocrine disruption in vertebrates and invertebrates and attempted to define this relatively new concept in the field of ecotoxicology (Appendix 1). More recently, León-Olea et al (2014) further developed this concept with specific examples, and identified key areas such as the links between neuroendocrine disruptors and disease and the implications of epigenetic and transgenerational effects of exposure to such pollutants.

1.2.2 Identification of neuroactive natural products

The secondary, and more exploratory, approach taken in this thesis was the potential for neuroendocrine activities identified in pulp mill effluents and their wood feedstocks to be used in the context of natural products research and value added products for the Canadian forest products industry. The neuroendocrine systems modulated by dopamine and γ-amino-butyric acid (GABA) play important roles in neurological disorders such as anxiety, epilepsy, Parkinson's disease, and depression in humans. The *in vitro* models (dopaminergic and GABAergic enzyme and receptor assays; see section 1.3.2 & 1.3.3) used in this thesis have important implications to such disorders.

1.3 Hypotheses and objectives

1.3.1 Hypotheses

The primary purpose of this thesis research was to determine if reproductive impairment in fish exposed to pulp mill effluents was linked to effects on neuroendocrine systems.

> **Hypothesis 1 (H1): Pulp mill effluents, their hardwood and conifer feedstocks, and phytochemicals found in various pulp mill effluent waste streams can disrupt key neurotransmitter systems implicated in the control of the reproductive axis in fish.**

The neurotransmitter systems that I examined for this research (reviewed in 1.3.2) are conserved in evolution, and also have important roles in neurological disorders and healthy brain function in humans. Any neuroactivities described in the vertebrate models tested for neuroendocrine disruption may therefore also have separate implications for natural product development.

> **Hypothesis 2 (H2): Chemicals in pulp mill effluents and their wood feedstocks can modulate the activities of neurotransmitter systems implicated in neurological disorders.**

The objectives outlined in each chapter of this thesis allowed me to support or refute H1 and H2.

1.3.2 Objectives

Objective 1: To measure milt production and ovulation in fathead minnows exposed to effluents that inhibit spawning.

Some pulp mill effluents rapidly and reversibly inhibit spawning in fathead minnows (Kovacs et al 2007). In Chapter 2, in close collaboration with Forest Product Innovations (FPInnovations, Pointe-Claire, QC, Canada), I provided data to help elucidate where in the reproductive axis inhibition of spawning is occurring. In the fish reproductive endocrine axis, a surge of luteinizing hormone (LH) release from the pituitary results in ovulation of eggs and an increase of milt production. In fish, this LH surge is under the direct control of the hypothalamus and telencephalon, also referred to as the neuroendocrine brain. If ovulated eggs and milt are indeed observed upon exposure of fathead minnows to an effluent that inhibits spawning, an appropriate LH surge is occurring and proper neuroendocrine function does exist, thereby refuting the neuroendocrine disruption hypothesis (H1).

Objective 2: To screen wood phytochemicals and extracts of feedstock wood species to identify potential neuroendocrine disruptors.

In Chapters 3 and 4, I assessed the potential neuroactivities of extracts from trees commonly used to produce pulp and paper as many of the compounds identified in pulp mill effluents and waste streams are phytochemicals from the wood feedstocks (Hewitt et al

2006). In Chapter 3, extracts from 14 North American hardwood species were tested for their ability to interfere with four neurotransmitter receptors and four enzymes important to proper neuroendocrine function. Furthermore, juglone, a phytochemical described in the neuroactive butternut tree (*Juglans cinerea*) extracts, was tested for the inhibition of two enzymes important for the synthesis and metabolism of key neurotransmitters involved in reproductive control and neurological disorders. In Chapter 4, extracts of 9 Canadian conifer species, as well as 16 phytochemicals implicated in pulp production, were tested in three neurochemical enzyme assays and one neurotransmitter receptor assay. These assays and associated neurotransmitter systems are described in sections 1.3.2 and 1.3.3. If phytochemicals and extracts of typical Canadian pulp mill feedstocks are active in neuroendocrine enzyme and receptor assays important for the control of the reproductive axis in fish, I will have identified an important potential source of neuroendocrine disruptors in pulp mill effluents and will thereby support the neuroendocrine disruption hypothesis (H1).

Objective 3: To assess the potential for phytochemicals and extracts of pulp mill feedstock tree species for neuropharmaceutical value as natural products.

The assays used in Chapters 3 and 4 also have important implications for neurological disorders. If bioactivities are observed in the screening of phytochemicals and extracts of wood feedstocks using these *in vitro* neuroendocrine assays, I will have supported the hypothesis that these extracts and compounds can modulate neuroendocrine function (H2).

Objective 4: To identify glutamic acid decarboxylase inhibitors in pulp mill effluents and the chemical characterization of inhibitory effluent extracts and fractions using advanced analytical chemistry techniques.

Glutamic acid decarboxylase (GAD) is an enzyme that synthesizes GABA, one of the key regulators of the reproductive axis that stimulates LH release (section 1.3.2). Ethylacetate extracts of balsam fir (*Abies balsamea*, the primary wood feedstock of the reproductively inhibitory mill effluent tested in Chapter 5), primary treated mill effluent, and final treated effluent were consistently inhibitory to GAD. This consensus of GAD inhibition

by ethylacetate fractions of wood feedstock and untreated and treated effluents indicated that bioactive compounds may be resistant to the various pulping and effluent treatment processes, therefore this assay was chosen for guiding chemical characterization. Inhibition of GAD activity by effluent extracts in Chapter 5 would support the neuroendocrine disruption hypothesis (H1) while the characterization of these effluent samples would help elucidate the active putatives responsible for this inhibition.

Objective 5: To identify glutamic acid decarboxylase inhibitors in the feedstock balsam fir and the chemical characterization of inhibitory balsam fir extracts and fractions.

Inhibition of GAD activity by balsam fir ethylacetate extracts and the phytochemical characterization of these extracts in Chapter 6 would support the hypothesis (H2) that natural compounds in feedstocks can modulate neuroendocrine disorders of the GABAergic system. Furthermore, identification of active putatives in a pulp feedstock species would help guide the search for neuroendocrine disruptors in effluents, thereby supporting H1.

Objective 6: To compare fractions inhibitory to glutamic acid decarboxylase from a pulp mill effluent and its primary feedstock using advanced analytical chemistry techniques.

In Chapter 6, I compared medium polar ethylacetate extracts and fractions of balsam fir and a pulp mill effluent where balsam fir is the primary feedstock using advanced metabolomics techniques with ultra-high-performance liquid chromatography time-of-flight mass spectrometry (UPLC-QTOF). The similarities I identified using advanced metabolomics would demonstrate that working with feedstock species can be an effective approach to identify bioactive principles present in treated effluents and support research in elucidating the neuroendocrine disruption hypothesis (H1).

1.4 Background and literature review

1.4.1 Pulp mill effluents disrupt reproduction in fish

The forest products industry has long been one of the most significant industries in Canada, and despite having lost over 86,000 jobs between 2006 – 2012, it contributed to nearly 2% of the nation's 2012 GDP. Of the roughly $26 billion dollars of the Canadian

forest products export market (which is second only to oil and gas), over $17 billion comes from pulp and paper (FPAC 2014). A typical pulp and paper mill may discharge over 100,000,000 litres of effluent daily. These effluents are a complex mixture of waste produced from debarking, pulp washing, bleaching, and regeneration of cooking chemicals. As the third largest pulp producer after the United States and China, Canada has over 150 pulp and paper mills emptying wastewater to aquatic systems. Care must be taken to ensure that impacts of these effluents to the Canadian environment are minimized. With new and expanding pulp and paper production capacity in South American and Asian countries, and long-established industries in Europe and North America, research on the effects of pulp and paper mill effluents on fish species has global significance.

Before 1992, effluents were linked to serious negative effects on fish survival and environmental health (Hewitt et al 2006). Public concern over the release of dioxins and acute fish toxicity led to increased federal regulation of pulp mill effluents under the *Canadian Environmental Protection Act* and the *Fisheries Act* in 1992. This required mills across the country to implement secondary treatment systems, eliminating the emission of dioxins, furans, and adsorbable organic halogens, as well as reducing total suspended solids and biochemical oxygen demand. These changes in effluent management led to the elimination of acute toxicity previously observed in wild fish populations.

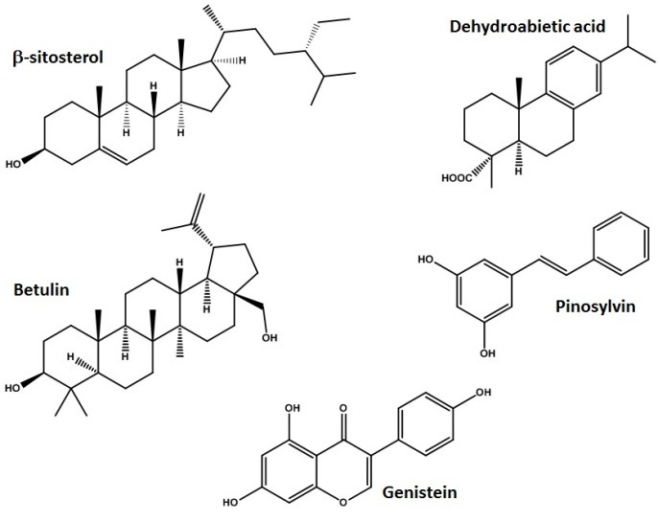


Figure 1.1 - Phytochemicals previously described in pulp mill effluent waste streams.

Despite the improvements seen in aquatic habitats and fish populations impacted by pulp and paper mills since the implementation of secondary treatment systems, many studies and the ongoing Environmental Effects Monitoring program of Environment Canada demonstrated that fish living in effluent-receiving waters were still

experiencing metabolic and reproductive disruption, namely decreased gonad size, increased liver size, and increased condition factor (McMaster et al 2006). Thus, endocrine disruption in fish exposed to pulp and paper mill effluents have been an important research focus for environmental toxicologists and endocrinologists for the last decade and a half (for reviews, see Hewitt et al 2006, McMaster et al 2006, Parrott et al 2006) with concerted efforts towards solutions (Hewitt et al 2008, Kovacs et al 2007, 2011, 2013, Martel et al 2011). This previous research led to the identification of numerous phytochemicals (Fig. 1.1), such as β-sitosterol, dehydroabietic acid, betulin, pinosylvin, and genistein, all of which have been implicated in pulp mill effluents and found to potentially impact normal reproductive endocrine function, although effective secondary treatment systems are capable of removing many of them from effluents (Christianson-Heiska et al 2008, Kiparissis et al 2001, Leusch & MacLatchy 2003, Mellanen et al 1996, Pelissero et al 1991, Tremblay & Van Der Kraak 1999). Furthermore, fathead minnows exposed to effluents in laboratory studies exhibit a rapid and reversible inhibition of spawning (Fig. 1.2) (Kovacs et al 2007).

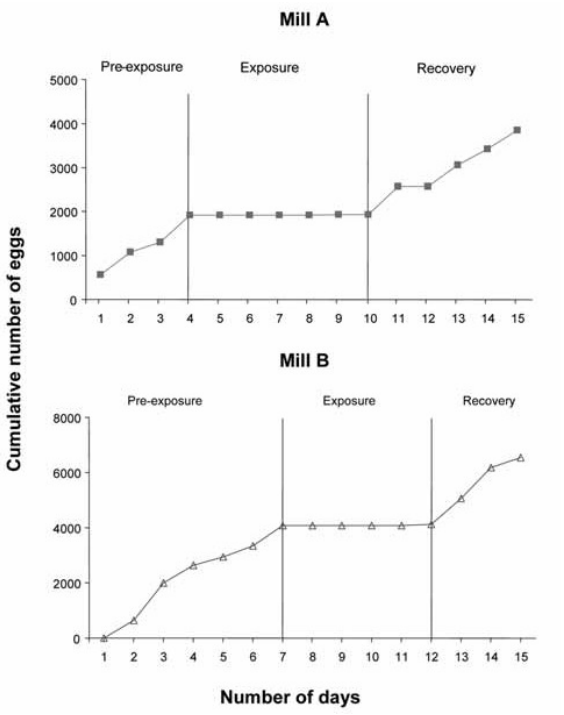


Figure 1.2 - Rapid and reversible inhibition of spawning in fathead minnow exposed to effluent (Kovacs et al 2007).

Disruption at the level of the brain has been an overlooked potential mechanism involved in these perturbations, whereby effluent chemicals may interfere with hypothalamic neurotransmitter and neuropeptide pathways critical for the regulation of pituitary and gonadal function (Basu et al 2009, Popesku et al 2008, 2010). This observation led to the development of the neuroendocrine disruption hypothesis for the actions of pulp and paper mill effluents to inhibit reproductive processes in fish (Popesku et al 2010, Waye & Trudeau 2011), first described in a paper I published with Basu et al (2009) (Appendix 2) and has further been explored outside of this thesis in

a paper I published with collaborators from Environment Canada during my doctoral research (Milestone et al 2012; Appendix 3).

1.4.2 Neuroendocrine control of reproduction in fish

In fish, follicle-stimulating hormone (FSH) and LH are the gonadotropic hormones released from the pituitary gland where they play important stimulatory roles in gonadal steroidogenesis and gametogenesis (Suzuki et al 1988, Swanson et al 1991). Moreover, a surge of LH causes sperm release and ovulation at the time of spawning (Peter & Yu 1997, Yaron 1995). The control of LH has been well studied in fish and stimulation is mostly under the control of the neuropeptergic gonadotropin-releasing hormone (GnRH) system. GnRH serves to coordinate and integrate neural and endocrine signals to regulate the synthesis and release of LH and, in teleosts, is primarily stimulated by γ-aminobutyric acid (GABA). Dopamine is responsible for the negative control of LH release, overriding the stimulatory effects of GnRH (Kah et al 1987, Peter et al 1986, Popesku et al 2008, Trudeau 1997).

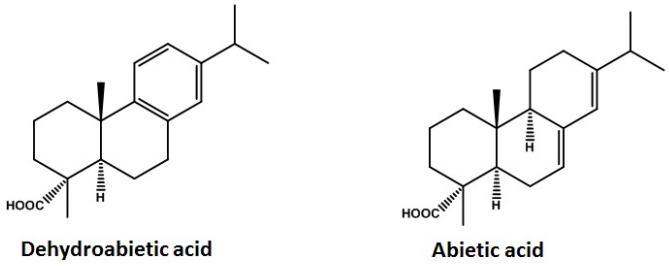


Figure 1.3 - Two common conifer resin acids described in pulp mill effluents

The research presented in my thesis follows the work by Basu et al (2009; Appendix 2), where we first described the potential for neuroendocrine disruption by pulp mill effluents, and that of Popesku et al (2010), where microarray analyses showed disruption of gene expression in the neuroendocrine brain of fathead minnows exposed to inhibitory effluents. Previous to the Basu et al (2009) and Popesku et al (2010) papers, only four other studies had directly implicated the effects of pulp mill effluents on the neuroendocrine axis. The first was by Van Der Kraak et al (1992), who demonstrated the effects of effluents on pituitary function. In this study, LH levels were lower in populations of white sucker (*Catostomus commersonii*) downstream of mill discharge. When these exposed fish were injected with a GnRH agonist, the LH surge and subsequent ovulation seen in control fish was not observed. The second and third studies were on dehydroabietic acid, abietic acid, and 12,14-dichlorodehydroabietic

acid, where Zheng & Nicholson (1996, 1998) were able to stimulate GABA release (Zheng & Nicholson 1996) and mobilize free [Ca^{2+}](Zheng & Nicholson 1998) from trout brain synaptosomes with these three compounds. In these papers, only the neurotoxic potential of these compounds was discussed and neuroendocrine effects were not considered. Dehydroabietic and abietic acid are two resin acid diterpenoids (Fig. 1.3) commonly found in conifer trees. They historically contributed to the acute toxicity of pulp mill effluents in fish before the adoption of secondary treatment systems at pulp mills. The chlorinated resin acid 12,14-dichlorodehydroabietic acid was a chemical by-product of pulp bleaching with elemental chlorine, a practice since abandoned by industry due to the production of dioxins and furans. In the fourth study, Nicholson et al (1999) demonstrated that 12,14-dichlorodehydroabietic acid inhibited GABA-stimulated chloride channels in mouse brain synaptosomes by irreversibly blocking ligands from binding to the convulsant site of the GABA type A receptor, demonstrating the potential to disrupt proper signaling in the central nervous system.

1.4.3 Model species, assays, and tested samples

In Chapter 2, I used fathead minnows as a model species for reproductive inhibition. An *in vivo* 5-day fathead minnow (*Pimephales promelas*) spawning assay is used by industry at the laboratories of FPInnovations to assess the quality of Canadian pulp and paper mill effluents and undertake investigation of cause studies (Kovacs et al 2007, 2013, Martel et al 2011). This assay was developed following multi-generational life-cycle studies with fathead minnows (FHM) where the effects of metabolic and reproductive disruption seen in wild fish populations exposed to effluents were successfully mimicked. During these life-cycle studies, the rapid and reversible inhibition of spawning was identified as a relatively quick and sensitive way to measure reproductive disruption by pulp mill effluents and the 3-week egg production assay designed by Ankley et al (2001) was adopted for monitoring effluent quality (Parrott 2005). The resulting 3-week FHM spawning assay was further optimized to a 5-day assay, where active effluents were observed to elicit rapid (overnight) and reversible spawning inhibition of breeding fathead minnows (Kovacs et al 2007). Through a partnership with FPInnovations, I designed an experiment by which I could assess the ability of females to ovulate and males to produce milt upon exposure to a thermo-mechanical pulp mill effluent that is a strong inhibitor to spawning.

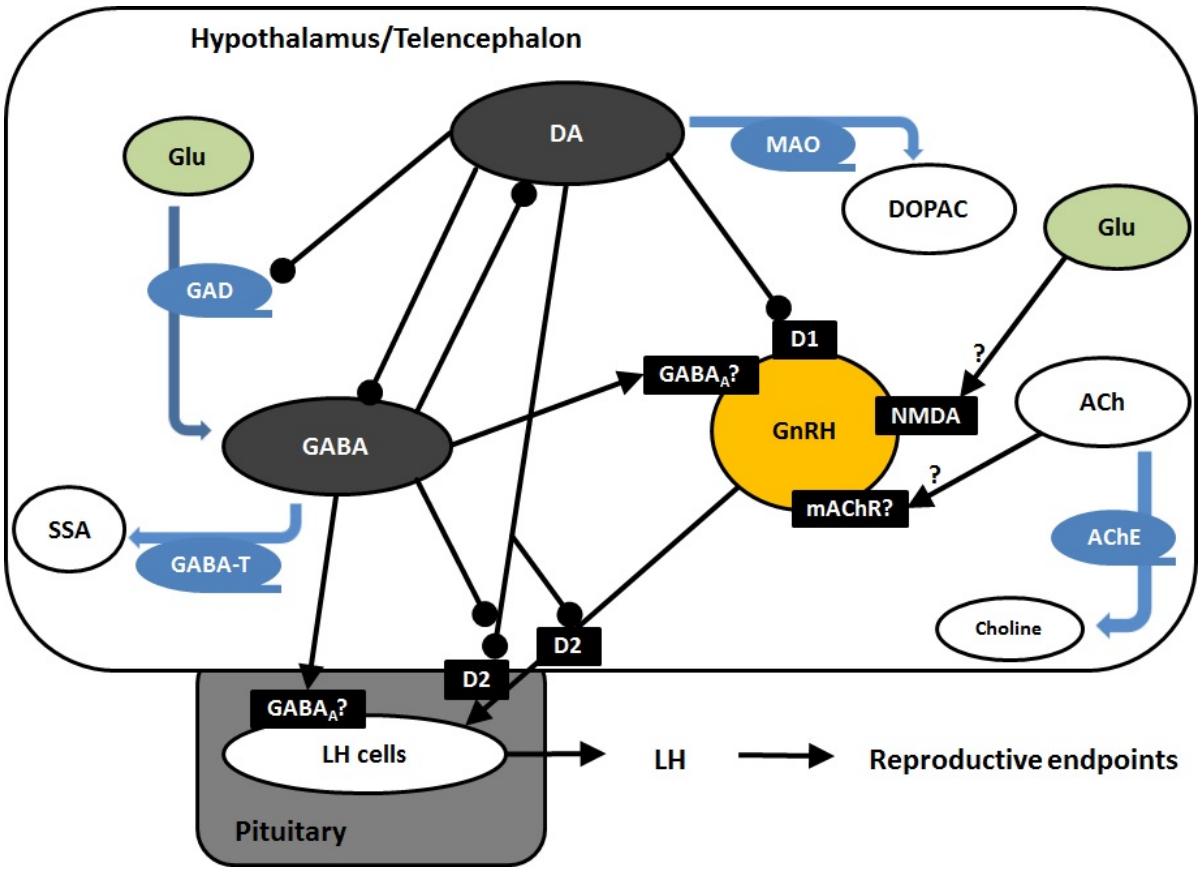


Figure 1.4 - Simplified model for the neuroendocrine control of reproduction in fish. Arrowed lines signify stimulation while circled lines signify inhibition. Black boxes represent receptors while curved arrows with attached graphics signify enzymatic reactions. Question marks exist where biological activity is not thoroughly characterized. DA, dopamine; MAO, monoamine oxidase; DOPAC, 3,4- dihydrophenylacetic acid; Glu, glutamic acid; GAD, glutamic acid decarboxylase; GABA, γ-aminobutyric acid; GABA-T, GABA transaminase; SSA, succinic semialdehyde; GABA(A), GABA type A receptor; D1, dopamine type 1 receptor; D2, dopamine type 2 receptor; GnRH, gonadotropin-releasing hormone; NMDA, N-methyl-D-aspartate receptor; mAChR, muscarinic acetylcholine receptor; ACh, acetylcholine; AChE, acetylcholinesterase; LH, luteinizing hormone.

In Chapters 3 – 6, I used *in vitro* neuroendocrine assays prepared from goldfish brains. The goldfish is an ideal model species for neuroendocrine function, as it has direct innervation of a highly regionalized pituitary (as opposed to delivery of neurohormones via the median eminence to a non-regionalized pituitary, as seen in mammals). The goldfish is large enough to easily dissect and handle while small enough to hold many in smaller facilities and is also a hardy and readily available organism. These traits have facilitated

detailed characterization of the neuroendocrine control of teleost reproduction. Figure 1.4 shows a simplified model for the neuroendocrine control of reproductive function as they relate to this thesis (reviewed by Popesku et al 2008, Trudeau 1997, Zohar et al 2010). Furthermore, as a member of the Cyprinidae (carp) family, the goldfish is representative of the largest families of vertebrates and many of the neurotransmitter receptors, enzymes, and hormones examined in this thesis have closely related mammalian orthologs (Popesku et al 2008). Therefore, there are implications for phytochemically-derived or natural health product treatments where these neuroendocrine systems are implicated, thereby lending goldfish not only as a model for neuroendocrine disruption of reproduction, but also as a model for potential natural product development for mammals (Chapters 3 and 4).

The *in vitro* assays used in this research focused mostly on the GABAergic and dopaminergic systems and mostly used fish brain tissues, but in Chapter 4, I more directly explored the pharmacological potential of boreal forest phytochemicals by including the GABA-transaminase (GABA-T) enzyme activity and GABA(A)-benzodiazepine (GABA(A)-BZD) receptor-binding assays (described later in this section) using rat (*Rattus norvegicus*) brain tissue. These two assays were established and optimized in our lab and used to screen botanicals for potential treatments of neurological disorders (Awad et al 2007, 2009). Neuroactive compounds identified in the assays performed with rat tissue would

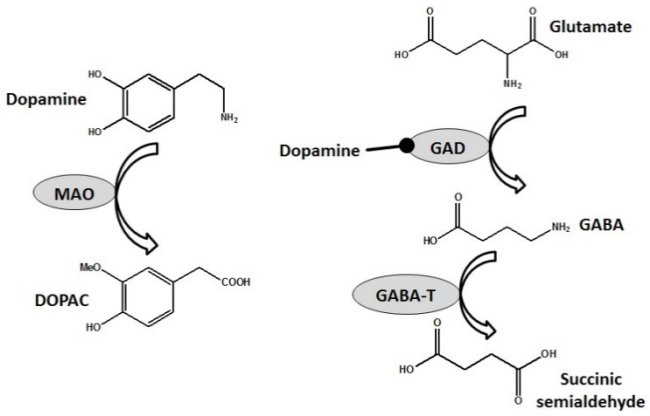


Figure 1.5 - Enzymatic reactions in the GABAergic and dopaminergic neuroendocrine systems. Curved arrows with graphics signify enzyme reaction and line ending with circle signifies inhibition. MAO, monamine oxidase; DOPAC3,4-dihydrophenylacetic acid; GAD, glutamic acid decarboxylase; GABA, γ-aminobutyric acid; GABA-T, GABA transaminase.

likely retain their ability to interact with the fish GABA-T enzyme and GABA(A)-BZD receptor due to the similarities in these proteins across vertebrates.

In Chapters 3 and 4, I screened tree extracts and phytochemicals in the GABA-T, GAD, and monoamine oxidase (MAO) enzyme assays (Fig. 1.5). The enzyme GABA-T is responsible for the metabolism of GABA to succinic semialdehyde. While changes to GABA-T function could lead to reproductive disruptions in fish as GABA is a potent stimulator of the LH release in goldfish (Popesku et al 2008, Trudeau 1997), any inhibitors of GABA-T that we identify could also have the potential for development as natural products in mammals. GABA is the major inhibitory neurotransmitter in the vertebrate central nervous system, and GABA-T inhibitors (e.g., the anti-epileptic pharmaceutical vigabatrin) are used to treat hyperactive nervous disorders by increasing GABA levels in the brain (Ashton & Young 2003, Sherif & Saleem Ahmed 1995). Phytochemical inhibitors of GABA-T have been previously studied in our lab for their potential to treat epilepsy and anxiety (Awad et al 2007, 2009). The enzyme GAD is responsible for the synthesis of GABA from glutamate and anxiolytic botanicals have been shown to inhibit GAD *in vitro* (Awad et al 2007). In fish, we would expect that any decreases of GABA levels due to an inhibition of GABA synthesis by GAD would result in an inhibition of the reproductive neuroendocrine axis, because GABA stimulates LH release and inhibits dopamine (Fig. 1.4), the potent inhibitor of the reproductive axis in many fish species (Kah et al 1987, Peter et al 1986, Trudeau 1997). Monoamine oxidase is one of the three enzymes responsible for the two-step metabolism of dopamine to homovanillic acid. The others are catechol-O-methyl transferase which methylates the 3' hydroxy group of dopamine and aldehyde dehydrogenase that (with MAO) replaces the amine with a carboxylic acid. Inhibitors of MAO injected into goldfish can increase dopamine levels in the pituitary as well as the hypothalamus and telencephalon, the two brain regions responsible for neuroendocrine control of the pituitary. These increased levels of dopamine can then lead to a subsequent decrease in serum LH (Sloley et al 1992). We would expect that MAO inhibitors identified in this thesis could lead to similar effects on LH, thereby providing a possible mechanism for the inhibition of the reproductive axis observed in lab and wild fish exposed to pulp mill effluents. In humans MAO inhibitors are used in the treatment of anxiety and depression and

can have neuroprotective effects against Parkinson's and Alzheimer's disease (Youdim 2010).

In Chapter 3, the acetylcholinesterase enzyme assay from goldfish brain preparations was also performed. This enzyme degrades acetylcholine present in synapses. Acetylcholine is stimulatory to the reproductive axis in mammals (Floody 2014, Justo et al 1975) and inhibition or stimulation of this enzyme might lead to disruptions to the reproductive axis of fish. Inhibitors for this enzyme are also used in venoms, nerve agents, insecticides, and the treatment of Alzheimer's disease symptoms (Colovic et al 2013). Receptor-binding assays performed in Chapter 3 included type 2 dopamine (D2) receptors, the GABA(A) receptor, the *N*-methyl-d-aspartic acid (NMDA) type of glutamate receptor, and the muscarinic cholinergic receptor. Ligands from samples that bind to D2 and GABA(A) receptors would have clear implications on the reproductive axis as their strong inhibitory and (respectively) stimulatory tones are mediated through these two receptors (Fig 1.4) (Popesku et al 2008). There is evidence that glutamate is stimulatory to GnRH in fish via the NMDA receptor (reviewed in Trudeau 1997, Trudeau et al 2000) and in mammals this receptor also plays an important role in synaptic plasticity and memory function (Li & Tsien 2009). Antagonists for the NMDA receptor, such as ketamine or nitrous oxide, are often used as recreational drugs for their hallucinogenic and dissociative effects and modulators of this receptor are being explored for the treatment of mood disorders such as depression (Lapidus et al 2013). The muscarinic cholinergic receptor is implicated in mammalian control of LH (Chiodera et al 1985, Krsmanovic et al 1998) and is an important pharmaceutical target for treatment of schizophrenia, Alzheimer's, and Parkinson's disease (Langmead et al 2008).

In Chapter 4, I used the GABA(A)-BZD assay. This binding site potentiates the activation of GABA(A) receptor by GABA (Smith & Olsen 1995), therefore I expect benzodiazepine-binding site agonists to be stimulatory to the reproductive axis in fish. From a pharmacological perspective, the benzodiazepine class of drugs are used to treat diverse neurological disorders such as depression, insomnia, and anxiety.

I screened both hardwoods and conifers for their ability to modulate neurotransmitter enzymes and receptors in fish and for their potential for natural product development. The hardwood species tested in Chapter 3 (Table 3.2) were collected from the holding yard of a

representative hardwood processing pulp and paper mill in Ontario that is now closed. These 14 species represent a diverse range of hardwood species, encompassing 11 genera, 8 families, and 6 orders of angiosperm eudicot trees. These are representative of most of the deciduous species found in the Great Lakes – St. Lawrence mixed deciduous forest. Hardwood trees have vessel elements and fibres in their wood while in conifer trees it is tracheids that both provide transport of water and mineral salts as well as the majority of the tree's structural support. While hardwoods are commonly used as a pulp feedstock in countries of warmer latitudes, in Canada pulp production is primarily from conifer wood feedstocks since the longer tracheids make them a prime choice for papermaking.

Conifers are largely evergreen gymnosperm trees and shrubs and often the dominant plants in boreal forests and cooler or mountainous areas. The nine species of conifers tested in Chapter 4 (Table 4.1) are comprised of two families, the Pinaceae and the Cupressaceae. The Pinaceae are the largest conifer family, including 220-250 species in 11 genera with the majority of these in temperate regions. They comprise the dominant family in boreal regions such as Canada, and thus are of important economic and ecological value.

Canadian plants, including many hardwood and conifers species, have a history of ethnobotanical uses by aboriginal populations in Canada that suggest neuroendocrine function, such as in the treatment of headaches, mental disorders (e.g., anxiety, depression, insomnia, fatigue, nervousness), nervous system disorders (e.g., convulsions, hyperactivity), "fainting and fits", and "craziness" (Arnason et al 1981, Leduc et al 2006, Uprety et al 2012).

The effluents tested on FHM in Chapter 2 and that I characterized and tested for GAD activity in Chapter 5 are both from thermomechanical pulp mills in Eastern Canada. In Chapter 2, the primary feedstocks were spruce. The pulp produced there is bleached with $Na_2S_2O_4$ and hydrogen peroxide and the secondary treatment process is by an air activated sludge in a single batch reactor (Table 2.1). The effluent studied in Chapter 5 used primarily balsam fir to produce pulp. The bleaching is performed with hydrosulfite and sodium sulfite and secondary treatment is by a 24 hour activated sludge (Table 5.1).

1.4.4 Identification of bioactive chemicals from extracts and the novel application of metabolomics in the field of pulp mill effluent studies

Classically, there are two approaches to the identification of bioactive components in extracts. The first approach is bioassay-guided fractionation, where extracts are fractionated, tested for bioactivity, and bioactive fractions are further fractionated and tested until fractions contain isolated single bioactive compounds which can then be identified, typically by nuclear magnetic resonance (NMR). This approach is applied in both Chapters 5 (with primary and secondary treated effluents) and 6 (with a bioactive balsam fir wood extract). The second approach is the targeted identification of compounds known or expected to be bioactive in analyzed samples. This approach is also used in Chapters 5 and 6 where we screen extracts from effluent and the balsam fir extract for bioactive compounds identified in Chapter 4. Each of these approaches has their disadvantages when working with dilute and chemically complex samples. When performing bioassay-guided isolations, the yields of single bioactive compounds must be sufficient such that after several rounds of fractionation, adequate material remains for further fractionation or identification by NMR. When performing a targeted analysis of bioactives in samples, it can be the case that the compounds being screened are not in the most active fractions/extracts and that other unknown chemicals are responsible for observed bioactivities. Both of these challenges were encountered in Chapters 5 and 6 due to the complex nature and low yields of the effluent and wood extracts I worked with. With the application of advanced software and analytical chemistry techniques, I attempted to overcome these difficulties by applying a novel metabolomics approach to sample analysis. With UPLC-QTOF and advanced chemical analysis using MassLynx software, I performed discriminant analyses to identify chemical markers common to bioactive effluent extracts (Chapter 5) and comparative analyses to support the concept that bioactive compounds in wood feedstocks are present in treated effluents (Chapter 6).

Chapter 2: Ovulation but not milt production is inhibited in fathead minnows (*Pimephales promelas*) exposed to a reproductively inhibitory pulp mill effluent

[1]Andrew Waye, [1]Wudu E Lado, [2]Pierre H Martel, [1]John T Arnason, [1]Vance L Trudeau

[1]Department of Biology, University of Ottawa, Ottawa, ON, K1N 6N5

[2]FPInnovations, Pointe-Claire, QC, H9R 3J9

This study was to test if ovulation and milt production were occurring in fathead minnows exposed to an effluent that inhibited spawning. If ovulation and milt production were observed, it would allow me to refute the neuroendocrine disruption hypothesis. This article has been published in Reproductive Biology and Endocrinology (Waye et al 2014b) with minor modifications for this thesis.

Statement of author contributions

AW, JTA, and VLT conceived the study, while method development and design was undertaken by AW, WEL, and PHM. AW performed study and data analyses. AW prepared manuscript with contributions from WEL, PHM, JTA, and VLT.

2.1 Introduction

In 1992 Canada initiated national Environmental Effect Monitoring (EEM) studies to measure the impacts of pulp and paper mill effluents on benthic invertebrates and adult fish. In the 20 years of EEM cycles, it has been identified that there is a national trend of larger livers and smaller gonads in fish living downstream of pulp mill effluents. Reproductive and metabolic disruption has therefore been a major research focus for environmental toxicologists and endocrinologists. While several specific chemicals have been identified, none have been convincingly demonstrated to be responsible for the effects observed in the wild (for reviews, see Hewitt et al 2006, McMaster et al 2006, Parrott et al 2006). Considerable effort has been made to identify solutions to the effects of effluent on wild populations (Hewitt et al 2008) and one result of these efforts was the development of an *in vivo* 5-day fathead minnow (*Pimephales promelas*) spawning assay that is now used by industry at the laboratories of Forest Product Innovations (FPInnovations) to assess the quality of Canadian pulp and paper mill discharge and guide investigation of cause studies (Kovacs et al 2007, Martel et al 2011). This assay was developed following multi-generational life-cycle studies with fathead minnows (FHM) where the effects of metabolic and reproductive disruption seen in wild fish populations exposed to effluents were successfully mimicked. During these laboratory life-cycle studies, the ability of fathead minnows to spawn was identified as a relatively quick and sensitive way to measure reproductive disruption by pulp mill effluents and the 3-week egg production assay designed by Ankley et al (2001) was adopted for monitoring effluent quality (Parrott 2005). The resulting 3-week FHM spawning assay was further optimized to a 5-day assay, where active effluents were able to elicit rapid (overnight) and reversible spawning inhibition of breeding fathead minnows (Kovacs et al 2007).

My on pulp mill effluents followed the hypothesis that pulp mill effluents contain neuroendocrine disruptors that inhibit reproductive processes in fish (Basu et al 2009, Waye & Trudeau 2011). Since ovulation and sperm release are controlled by a neuroendocrine cascade, I hypothesized that such a rapid and reversible inhibition of spawning may be neuroendocrine in nature (for example, perhaps by disrupting the luteinizing hormone (LH) surge necessary to trigger spawning) (Waye & Trudeau 2011). In the following chapters of this thesis, and through published collaborations, I have performed assays to demonstrate

that effluents and wood feedstock used by mills contain potentially neuroactive chemicals that can act on the dopamine and GABA systems (Basu et al 2009, 2012, Milestone et al 2012, Waye et al 2014a), as they are two important systems that modulate LH release (Peter et al 1986, Popesku et al 2008, Trudeau 1997).

The objective of the present study was to determine whether it is the males or the females whose ability to spawn is inhibited. More specifically, I tested whether ovulation in females and/or milt production in males were affected by exposure to an inhibitory effluent. If ovulation and milt production are observed after exposure, I could refute my hypothesis that spawning inhibition is neuroendocrine in nature (Basu et al 2009, Waye & Trudeau 2011) and that the pituitary is not able to produce an LH surge. If an inhibition of ovulation and/or milt production is observed, then the disruption of the neuroendocrine control of reproduction hypothesis is still valid, although other mechanisms (e.g., at the level of the gonad) may also be at play.

2.2 Methods

2.2.1 Pulp mill description and parameters

Effluent samples were from a thermomechanical pulp (TMP) mill in Eastern Canada that produces 889 t/d of newsprint. Effluent flow is 32 m^3/t of pulp (Table 2.1). Pulp is produced using wood comprised of 70-75% spruce and 25-30% fir as well as deinked pulp and is bleached with sodium hydrosulphite and hydrogen peroxide. Treatment of the effluent occurs using flotation units (15-20% of effluent flow), and secondary treatment comprises of air activated sludge in a sequential batch reactor.

2.2.2 Sample collection and storage

Grab samples were taken from the final effluent outflow on May 22, 2012 and shipped in two 1000 L bulk containers lined with food-grade polyethylene to FPInnovations in Pointe-Claire, Québec, for fathead minnow exposures. Upon arrival, contents were transferred to 210 L polyethylene barrels and stored at 4^o Celsius. Exposures began on May 25, 2012 and were terminated on May 30, 2012. The effluent sample was analyzed for pH (Orion Model 1230, Thermo Fisher Scientific, Ottawa, ON, Canada), conductivity (Orion Model 162A, Thermo Fisher Scientific, Ottawa, ON, Canada), dissolved oxygen (YSI 52,

Table 2.1 Pulp mill description and operating procedures.

Pulping	Brightening agent	Furnish	Product	Production	Water usage	Primary treatment	Secondary treatment
TMP deinked pulp	$Na_2S_2O_4$ / H_2O_2	25-30% fir, 70-75% spruce Deinked pulp: > 80% ONP	Newsprint	889 t/d	32 m^3/t	Flotation[1]	SBR

[1] Treats only 15-20% of primary effluent flow in flotation units.
TMP, thermomechanical pulp; ONP, old newspaper; SBR, air activated sludge in sequential batch reactor.

Table 2.2 Measured physicochemical parameters of tested effluents.

Date sampled	pH	Conductivity	Hardness	DO	NH_3	BOD	COD	SS	RFA
May 22, 2012	7.2	2060 µS	918 mg/L	4.8 mg/L	0.1 mg/L	10 mg/L	187 mg/L	9.9 mg/L	0.01 mg/L

DO, dissolved oxygen; BOD, biological oxygen demand; COD, chemical oxygen demand; SS, suspended solids; RFA, resin and fatty acids (detection limit 0.01 mg/L).

Yellow Springs Instruments Inc., Yellow Springs, OH, USA), and ammonia (Accumet 950, Fisher Scientific, Ottawa, ON, Canada). Effluent biochemical oxygen demand, chemical oxygen demand, hardness, and total suspended solids were measured as per American Public Health Association methodology (Eaton et al 2005) and resin and fatty acids analysis was performed by gas chromatography (Voss & Rapsomatiotis 1985). Measured physicochemical parameters are described in Table 2.2.

2.2.3 Fathead minnow reproduction assays and effluent exposures

Fish were bred, cultured, selected, and cared for at a fathead minnow colony at the wet labs of FPInnovations (Pointe-Claire, QC, Canada) and the exposure regime occurred according to previously published methods (Kovacs et al 2007). Briefly, two males and four females were held in 12.5 L aquariums for a pre-exposure period of 7 days. Aquariums contained spawning substrates made of two 8 cm lengths of food-grade polyvinyl pipes with a 10 cm diameter cut in half longitudinally which were monitored in the mornings for daily egg production. Successful fertilization of spawned eggs was confirmed by observations using a dissection microscope. Groups exhibiting the highest reproductive performance were selected for the experiment.

Effluent exposures began on May 25, 2012 at a concentration of 100%. This concentration is the standard for industrial testing of effluents and investigation of cause studies (Martel et al 2011). At this concentration nearly complete spawning inhibition and no mortality was observed. Each treatment (control, 100% effluent, control stripped, and 100% effluent stripped) was performed with 4 replicate tanks. For the experiment and pre-exposure period, the replicates were kept in glass 12.5 L tanks under flow-through conditions with 4 to 6 tank volume renewals per day and were aerated at a minimum of 6.5 ml/min/L. The photoperiod used was 16 hours light and 8 hours dark. Each day tank pH (7.52 to 8.27), dissolved oxygen (>74.9%), and temperature (25^{o} C +/- 1^{o} C) were monitored and the spawning substrates were checked for egg production. For the stripped FHM in 100% effluent, fish were stripped just prior to effluent exposure, and on the third and last day (day five) of the experiment. All fish were sacrificed upon termination of experiment on day five. Snout to fork body length and wet body weight and gonad weight were recorded.

Fish were anaesthetized in a solution of 100 mg/L MS-222 (Sigma-Aldrich, Toronto, ON, Canada) and females were stripped of ovulated eggs and males were stripped of milt just before the initiation of the experiment (T = 0) and at days 3 and 5 (end of exposure). To strip eggs, anaesthetized females were held gently in the hand and the abdomen was massaged lightly using strokes of the thumb towards the posterior. Eggs released from the oviduct were collected with an eye dropper, weighed, and put aside for counting. To strip milt, males were similarly held and massaged and milt was collected in Fisherbrand Micro-Hematocrit Capillary Tubes (Fisher Scientific, Pittsburg, PA, USA) by suction applied via a tube connected to the mouth and subsequently weighed. When males were stripped of milt upon completion of the experiment, milt weight was added to gonad weight to correct to unstripped male gonadal weight.

2.2.4 Statistical analyses

Male and female length, weight, gonadosomatic index (GSI; Fig. 2.1), condition factor ($K = [\text{wet weight(g)}/\text{fork length(cm)}^3] \times 100$; Fig. 2.2), number of eggs spawned per tank (Fig. 2.3), total eggs produced per tank (Fig. 2.4), and male milt production (Fig. 2.5) were analyzed using two-way ANOVA (for stripping and effluent effects) and checked for normality and homoscedasticity (Levene's test). All statistics were performed using SPSS v. 17.0 (IBM, San Jose, CA, USA) and figures were created in GraphPad Prism v. 5.01 (GraphPad Software, La Jolla, CA, USA). Neither GSI nor K were covariates of milt or egg production.

2.3 Results

2.3.1 Morphometric parameters

Neither effluent exposure nor stripping had an effect on mean fish fork length or weight during the course of the exposure. For GSI, an effect on milt levels was observed from stripping, resulting in lower GSI in stripped males compared to unstripped males (Fig. 2.1b; $p < 0.001$). No effects were observed from effluent exposure compared to controls for both stripped and unhandled groups. No significant differences were detected for female GSI measurements (Fig. 2.1a). A difference in K was detected between stripped exposed (mean K 1.42 +/- 0. 0695% C.I.) and unstripped exposed (mean K 1.30 +/- 0.05 95% C.I.) females, but not when compared to controls (Fig. 2.2a).

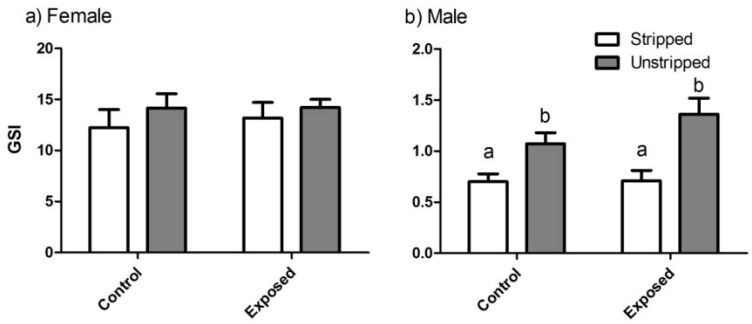


Figure 2.1 - Mean female (a; n=16) and male (b; n=8) gonadosomatic index (GSI). The GSI of white bars (which represent control or exposed fish that were stripped of ovulated eggs or milt) was calculated by adding gonad weight and weight of stripped milt or eggs. Grey bars represent control or exposed fish that were not handled. Error bars represent 1 standard error of the mean and significant differences denoted by differing letters ($p < 0.05$).

2.3.2 Egg production in female fathead minnows

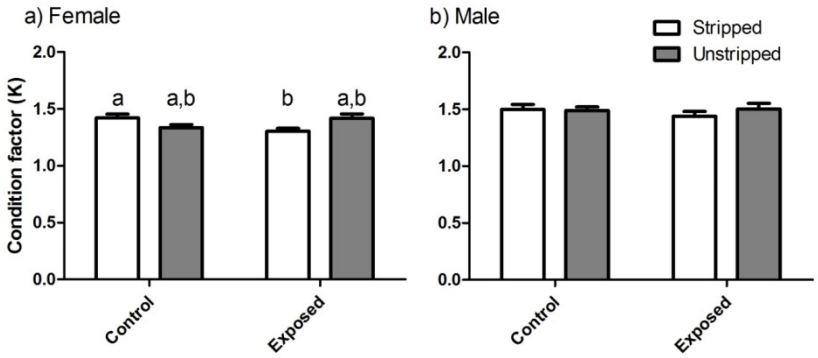


Figure 2.2 - Female (a; n=16) and male (b; n=8) condition factor (K). White bars represent control or exposed fish that were stripped of ovulated eggs or milt while grey bars represent control or exposed fish that were not handled. Error bars represent 1 standard error of the mean and significant differences denoted by differing letters ($p < 0.05$).

There were no significant differences in mean cumulative egg production ($p > 0.05$) between pre-exposure treatment groups, and all replicates spawned successfully (i.e., there were some eggs that were successfully fertilized) over the seven day pre-exposure period and over the duration of the experimental exposure.

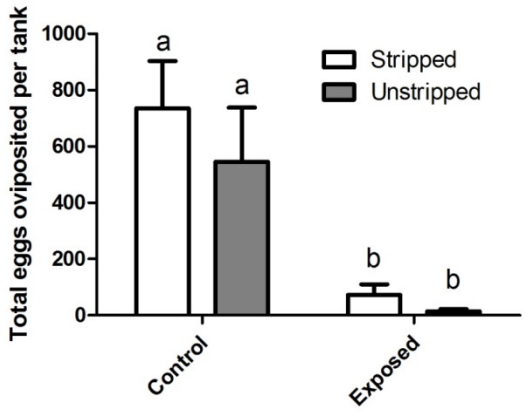


Figure 2.3 - Cumulative number of eggs oviposited per tank. White bars represent control or exposed tanks where fish that were stripped of ovulated eggs or milt while grey bars represent control or exposed tanks where fish that were not handled. Error bars represent 1 standard error of the mean and significant differences denoted by differing letters ($p < 0.05$; n=4).

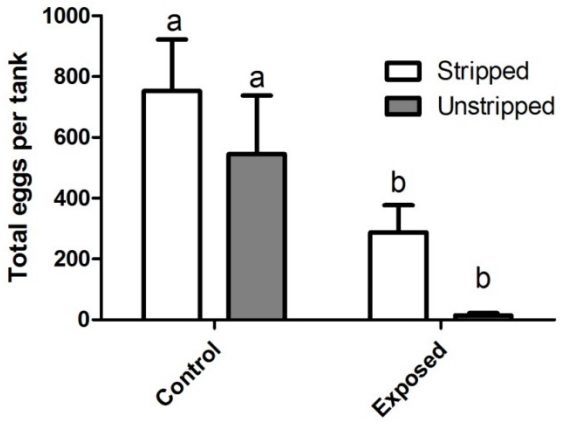


Figure 2.4 - Cumulative number of total eggs produced (stripped + oviposited) per tank. White bars represent control or exposed tanks where fish that were stripped of ovulated eggs or milt while grey bars represent control or exposed tanks where fish that were not handled. Error bars represent 1 standard error of the mean and significant differences denoted by differing letters ($p < 0.05$; n=4).

Exposure to the 100% TMP effluent caused a significant ($p < 0.05$) inhibition of spawning (Fig. 2.3). Untreated female groups oviposited the statistically same numbers of eggs regardless of stripping as did the effluent-exposed groups ($p > 0.05$; Fig. 2.3). When comparing the total numbers of eggs (oviposited + stripped), we observed that the total egg production in untreated groups was the same, regardless of stripping, as was the total egg production in the effluent exposed groups (Fig. 2.4; $p > 0.05$). The difference in total egg production between unstripped control and exposed groups was significant ($p < 0.05$) as was the difference between the stripped control and effluent exposed groups (Fig. 2.4). There was no significant effect of stripping the fish, nor was there a significant effect of the interaction between effluent exposure and stripping ($p > 0.05$).

2.3.3 Milt production in male fathead minnows

There was no significant difference ($p > 0.05$) between the amount of milt stripped from the control males and the males exposed to 100% effluent at any of the time points where milt was sampled (at T = 0 and after days 3 and 5 of exposure; Fig. 2.5).

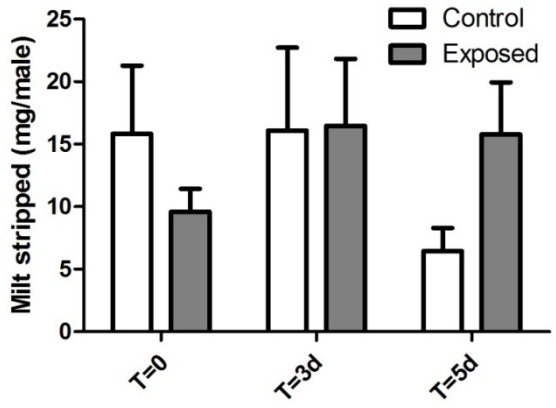


Figure 2.5 - Weight of milt stripped per male fathead minnow. White bars represent control tanks while grey bars represent exposure to pulp mill effluent. Error bars represent 1 standard error of the mean and no significant differences are observed ($p > 0.05$; n=8).

2.4 Discussion

2.4.1 Morphometric parameters

I did not observe any differences in morphometric parameters during the experiment with the exception of male GSI (Fig. 2.1b) and female K (Fig. 2.2a). I observed that GSI in males was lower at the end of the experiment in males that had been stripped of milt during the experiment. This may be explained by an effect of handling and stripping on the males resulting in higher demands on the gonad for milt (and thus a reduced gonad weight) when compared to unstripped fish. The difference detected in female K was likely due to slightly, but not significant ($p = 0.0564$), longer mean female fork length in the exposed stripped tanks. Changes in morphometric parameters were not observed during the shortened 5-day fathead minnow spawning assay in the study by Kovacs et al (2007). In the 5-day study of seven mechanical pulp mill effluents tested in the 5-day FHM spawning assay, an increased gonad weight from two mill effluent exposures was the only morphometric parameter observed to change (Kovacs et al 2013), while in a study of seven Kraft mill effluents, female body weight was lower in only one treatment (Kovacs et al 2011). The authors of these studies stated that these differences did not indicate effluent-related effects since measurements were taken at the end of the experiment after the fish had been removed from the effluents and kept in well water for five days. In the longer 21-day assay, no changes in morphometric parameters were seen in males but in females mean fork-length was lower in

one of the treatments (a 2% Kraft mill effluent) while mean weight and condition factor was higher in another treatment (a different Kraft mill effluent at 40% concentration) (Kovacs et al 2005). The short exposure period and variability of these effects across studies and those seen in the present study suggest that differences in measured morphometric parameters are likely artifacts of the randomized selection of individuals for each treatment group.

2.4.2 Egg production in female fathead minnows

Fewer eggs were laid in the female groups exposed to the TMP effluent. In order to assess if the females were not ovipositing despite ovulating, I stripped ovulated eggs from the exposed females. Reasons for females ovulating but not spawning might include males not detecting the release of pre- and post-ovulatory pheromones due to pheromone binding/adsorption to effluent constituents or blockage of male olfactory receptors resulting in male failure to initiate spawning behavior. Another reason may be that ovulated females are able to detect non-ideal conditions for their eggs and decide to forgo spawning until conditions improve. The reduced eggs laid and stripped in exposed tanks (Fig. 2.4) refute these scenarios, clearly demonstrating that ovulation is impaired in effluent-exposed fish compared to controls. If the exposed females were indeed ovulating, I would expect the total eggs per tank (oviposited + stripped) to be similar to the total eggs in the control tanks, only with a higher proportion of the total eggs being stripped, rather than laid, compared to controls.

In teleost fish, ovulation is triggered by a surge in LH from the pituitary, which is under the direct stimulation of gonadotropin-releasing hormone (Peter 1982). Dopamine and GABA are very important neurotransmitters in the reproductive axis because they respectively inhibit and stimulate LH release (Dufour et al 2010, Popesku et al 2008, Trudeau 1997). This inhibitory input by dopamine is potent, such that co-injection of a dopamine antagonist with a GnRH agonist is required to induce the LH surge and spawning in teleosts (Peter et al 1986). *In vitro* experiments on effluent extracts (Basu et al. 2009; Appendix 2) identified that extracts of a Canadian TMP effluent contained ligands for the dopamine type-2 receptor among other neuroendocrine targets important to reproductive control. In follow-up publications, I identified several effluents (Milestone et al 2012)

(Appendix 3) and hardwood (Basu et al 2012) (Chapter 3) and conifer (Waye et al 2014a) (Chapter 4) feedstocks that also have potential neuroactivities.

It is not possible to measure LH in FHM because the LH radioimmunoassay has not been developed for this species. Nevertheless, I hypothesize that LH release may be reduced such that the female FHM exposed to the pulp mill effluents were unable to ovulate. Prior to my work, a study by Van Der Kraak et al (1992) on a white sucker (*Catostomus commersoni*) population exposed to a bleached Kraft mill effluent from a mill in Terrace Bay, ON, Canada, clearly demonstrated that LH and sex steroids in exposed wild white suckers were depressed compared to those from control sites. Upon injecting females with a GnRH agonist, the size of the resulting LH surge observed in control fish was not seen in those populations exposed to effluents, nor did ovulation occur (while it did in controls). This indicated that either the pituitary had lost GnRH sensitivity (due to lesions or perhaps other mechanisms) or that other inhibitory signals (e.g., dopamine) might be suppressing LH release.

It is also possible that while an LH surge is indeed occurring in the females exposed in this study, the ovaries of these individuals may not be responding appropriately, but this is speculative because we did not directly measure steroid products. *In vitro* studies (Gibbons et al 1998, McMaster et al 1996) have previously demonstrated an impaired induction of steroidogenesis in the gonadal tissue of fish exposed to pulp mill effluents. Both testosterone and 17β-estradiol production was inhibited in ovarian follicles stimulated by human chorionic gonadotropin in tissues collected from wild white sucker (*Catostomus commersonii*) downstream of a sulphite pulp mill (McMaster et al 1996), and forskolin-stimulated 17β-estradiol production was inhibited in ovarian follicles collected from trout-perch (*Percopsis omiscomaycus*) downstream of a thermomechanical/de-inked pulp mill (Gibbons et al 1998). However, the significance of inhibited steroid production is unclear since previous studies have demonstrated that reduced spawning rates in fish exposed to effluents do not appear to be associated with several steroidogenic endpoints (Van den Heuvel et al 2010).

Additional mechanisms of action for spawning inhibition by effluents exist that may help explain the results seen in this chapter. For example, inhibition of steroidogenic brain

aromatase or 3β-hydrosteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD) is also able to elicit rapid inhibition of spawning in fathead minnows. The aromatase inhibitor fadrozole resulted in decreased brain aromatase activity in fathead minnows and in these fish, rapid spawning inhibition and impairment of oocyte maturation was observed (Ankley et al 2002). Plasma estradiol and vitellogenin concentrations were also decreased in these females exposed to fadrozole. Inhibition of 3β-HSD by trilostane also caused a rapid inhibition of spawning in fathead minnows as well as decreased levels of vitellogenin (Villeneuve et al 2008), so the observed effects in our study could be explained by effects at the level of the gonad or liver in addition to effects on brain. However, many effluents are strongly anti-reproductive, but inconsistently affect steroidal pathways (Munkittrick et al 1998, Wartman et al 2009). It is also possible that effluents are inducing a stress response and that increased cortisol levels play a part in the inhibition of ovulation, but there is little evidence for stress induction by effluents as measured by cortisol in fish (Linton et al 2005, McMaster et al 2006). Regardless of the hormonal response, pulp effluents are acting somewhere in the hypothalamus-pituitary-gonad axis and it is more than likely that this includes effects at all three levels; my results did not refute the possibility of reproductive disruption at the level of the neuroendocrine brain.

2.4.3 Milt production in male fathead minnows

I observed no effects from exposure to effluent on male milt production (Fig. 2.5), indicating that the reproductive inhibition is a female rather than a male effect. Males are likely detecting sex pheromones that are released by females to signal to males that they have ovulated, since the males are producing milt.

Typically, in female cyprinid fish the LH surge is followed shortly thereafter by the release of a pre-ovulatory hormone (17α,20β-P or an analog) from the somatic follicle cells in the ovary, which immediately precedes ovulation. This pre-ovulatory hormone is released to the environment as a sex pheromone and is detected by males. Upon detection, the males also experience an increase in LH and within hours milt volume begins to increase (for review, see Stacey and Sorenson 2005). While we conclude that this TMP effluent inhibits ovulation in female fathead minnows, it is evident that a few eggs are still ovulated and oviposited (Fig. 2.3 and 2.4). On day 3 of the experiment, we were able to strip eggs from 8

of the 16 females in the effluent treatment and on day 5, we were again able to strip eggs from 8 of the 16 exposed females. Since some females were still able to ovulate a few eggs in the effluent-treated tanks, it seems that the pre-ovulatory hormone released from these females was sufficient for the sensitive pheromonal detection by males as demonstrated by an appropriate milt production response.

These sex-specific effects on brain function were also observed in another study where fathead minnows were exposed to five inhibitory secondary-treated effluents in the 5-day fathead minnow spawning assay, and these differences were neuroendocrine in nature (Popesku et al 2010). Urotensin 1 and RevErbβ2 mRNA levels were preferentially affected in the neuroendocrine brain of females compared to males. Only one effluent decreased urotensin 1 mRNA in male hypothalamus, while all five effluents caused a decrease in females. In the telencephalon, one of the effluents caused an increase in urotensin 1 mRNA levels in females, while no changes were observed in males. For RevErbβ2, four of the five effluents resulted in different mRNA levels in the hypothalamus than controls (three decreased, one increased), while in males, only one of the five effluents caused a decrease in relative mRNA expression. RevErbβ2 is a nuclear receptor that plays an important role in circadian rhythms (Liu et al 2008). Since photoperiod is a strong determinant of reproductive success in fathead minnow (Clayton 1997), a shift in RevErbβ2 expression in the brain may be a mechanism by which spawning is disrupted. Another study also identified sex-specific effects on liver gene expression in fathead minnows exposed to secondary treated pulp mill effluents (Werner et al 2010). While no changes in gene expression was observed in female livers, males had increased expression of androgen receptor, estrogen receptor β, and cytochrome P4501A, although these effluents did not inhibit spawning during a 6-day exposure at 25% v/v effluent concentration (Werner et al 2010).

2.5 Conclusions

In this chapter, I show that exposure to an inhibitory TMP effluent in the 5-day fathead minnow spawning assay prevented ovulation in females, but not milt production in males. This result has several implications into the mechanistic effects of effluents that inhibit spawning. Research on inhibitory neuroendocrine pathways in the reproductive axis, or on the ability of the gonad and pituitary to detect and respond to signals that originate in

neuroendocrine pathways, could identify the mechanism by which ovulation is inhibited. Furthermore, milt production in males was not different in males exposed to this effluent when compared to controls, indicating that males likely remain sensitive to female reproductive pheromonal signaling.

The fathead minnow continues to be an amenable model for ecotoxicology and endocrine disruption research, and future focus on the mechanism behind the female-specific effect observed in this study could allow for the development of faster and more cost-effective assays by which to investigate environmental contaminants and their sex-specific reproductive effects. In this way, effective mitigations strategies may also be developed.

Chapter 3: Extracts from hardwood trees used in commercial paper mills contain biologically active neurochemical disruptors

[1]Nil Basu, [1]Andrew Waye, [1]Vance L Trudeau, [1]John T Arnason

[1]Department of Biology, University of Ottawa, Ottawa, ON, K1N 6N5

This study followed work in Basu et al (2009) where neuroactive components were found in pulp mill effluents, and tests whether the described neuroactivities are present in hardwood pulp and paper mill feedstock species, as phytochemicals originating in feedstocks may be present in mill effluents. This article has been published in Science of the Total Environment (Basu et al 2012) with minor modifications for this thesis.

Statement of author contributions

NB, VLT, and JTA conceived and designed this study. NB prepared samples and NB and AW performed enzyme assays. AW performed data analysis and prepared the manuscript with contributions from NB, VLT, and JTA.

3.1 Introduction

In a previous study I hypothesized that bioactive compound(s) within pulp and paper mill effluents can interact with, and disrupt, various neurotransmitter receptors and enzymes important to fish reproduction (Basu et al 2009). In that investigation, grab samples of primary- and secondary-treated effluents from a representative newsprint mill were fractionated using two established approaches (solvent series and polyphenolic extraction). The resulting extracts were subsequently screened for their ability to interact with key enzymes and receptors involved in neurotransmission by means of *in vitro*, competitive assays using goldfish (*Carassius auratus*) brain tissues, which are an excellent model for studying vertebrate neuroendocrine signaling (Popesku et al 2008). Radioligand binding to neurotransmitter receptors were significantly changed following *in vitro* incubations with extracts, with the dopamine type 2 receptor (D2) showing a 21−48% increase, the γ-aminobutyric acid type A receptor (GABA(A)) a 65−67% decrease, the *N*-methyl-D-aspartic acid receptor (NMDA) a 26−75% decrease, and the muscarinic cholinergic receptor (mACh-R) a 42% increase in binding. Activities of neurotransmitter-related enzymes were significantly altered as well, with monoamine oxidase (MAO) activity decreased 14−48%, GABA transaminase (GABA-T) activity 33% decreased and 21−69% increased, and acetylcholinesterase (AChE) 21−50% decreased. No changes in glutamic acid decarboxylase (GAD) activity were detected (Basu et al 2009). These findings provided a novel mechanism by which pulp and paper mills effluents may impair fish reproduction by interacting with key neurotransmitter systems to cause neuroendocrine disruption.

The presence of a large number of wood-derived natural products in pulp mill effluents (Hewitt et al 2006) suggests that neurochemical activity may also be found in the wood feedstocks of these mills. Plants, including trees, are a source of biologically active secondary metabolites and many medicinal plants affecting key neurotransmitter receptors and related enzymes are already well known (Heinrich et al 2008, Lakhan and Vieira 2010). They have been extensively studied for their hallucinogenic effects, and for their therapeutic potential for the treatment of depression and anxiety (Lakhan and Vieira 2010). However, the possible existence of neuroactive substances in temperate tree species is less studied. In the present study we examined the neurochemical activity of extracts of a group of hardwood logs sampled at a commercial pulp and paper facility. While this work has implications for

effects on fish and other biota downstream of pulp and paper mills, it also provides information on new sources of biologically active natural products which may be leads for medicinal products or for further study of the chemical ecology of plant herbivore interactions.

3.2 Materials and methods

3.2.1 Pulp and paper feedstock logs and preparation of extracts

Unprocessed raw logs of 15 hardwood species were sampled from the holding yard at a representative Ontario pulp mill in 2000. Bark and wood were extracted in 80% ethanol as described in Omar et al (2000). Details including voucher numbers are provided in the Omar et al (2000) paper. As the purpose of this study was to determine if neuroactive components exist in pulp and paper feedstocks, all dried extracts were resuspended in 80% ethanol and bioassayed at 0.5 mg/ml. Testing a fixed concentration of the sample enabled direct comparisons to be made among all extracts. Juglone, a phytochemical common in butternut, was provided by the Arnason lab phytochemical inventory at the University of Ottawa (Ottawa, ON, Canada).

3.2.2 Preparation of goldfish brain tissues

The *in vitro* experiments were carried out on whole brain tissues pooled from ~100 common goldfish (*Carassius auratus*). For the receptor binding studies, cellular membranes were isolated and prepared by homogenizing tissues in 1:10 volumes of buffer (see Table 3.1 for specific details) according to established methods (Basu et al 2009). The homogenate was centrifuged twice (32,000x*g*, 15 min, 4 ºC) and the resulting pellets were rinsed in buffer. The final pellet was re-suspended in buffer and aliquots of cellular membranes were immediately frozen to –80 °C until required. For MAO and AChE assays, brain tissues were sonicated in cold Na/K buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4) that included 0.5% (*v/v*) Triton X-100 for 30 sec. Next, the sample was centrifuged at 15,000x*g* (4ºC) for 10 min and then the supernatant was removed and stored at –80 °C until assayed. For GAD assays, tissues were homogenized in 1:10 (w/v) of phosphate buffer (pH 7.5). The homogenate was stored at –80 °C until analysis. For GABA-T assays, brains were homogenized in 1:10 buffer (20% glycerol, 0.13% Triton X-100, 0.1 mM reduced

Table 3.1. Methodological aspects for neurochemical receptor binding assays.

Neurochemical receptor	Buffer[a]	Pre-treatment of microplates[b]	Radioligand, specific activity[c]	Displacer information[d]	Incubation condition
Dopamine-2 (D2)	50 mM Tris HCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$	100 µl of 0.1% polyethylenimine	[^{3}H]-spiperone, 15.7 Ci/mmol, 5 nM	(+)-butaclamol	90 min, 25 °C
GABA(A)	50 mM Tris HCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$	100 µl of 0.1% polyethylenimine	[^{3}H]-muscimol, 18 Ci/mmol, 32 nM	muscimol	30 min, 4 °C
NMDA	50 mM Tris, 100 µM glycine, 100 µM L-glutamic acid	100 µl of indicated Tris buffer	[^{3}H]-MK801, 22 Ci/mmol, 5 nM	MK-801	120 min, 25 °C
mAChR	50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl	100 µl of indicated Na/K buffer	[^{3}H]-QNB, 42 Ci/mmol, 1 nM	atropine sulfate	60 min, 25 °C

[a] Buffers were adjusted to pH 7.4. [b] Plates were pre-conditioned for 30 min with 100 µL of buffer or 0.1 % polyethylenimine (PE). [c] Radioligands were purchased from NEN/Perkin Elmer (Boston, MA, USA). [d] Displacers were tested at 100 µM. GABA(A), g-aminobutyric acid type A receptor; NMDA, N-methyl-D-aspartate receptor; mAChR, muscarinic acetylcholine receptor.

glutathione, 0.1 mM pyridoxal-5'-phosphate, 1 mM Na_2EDTA, 10 mM K_2HPO_4, pH 6.8), and centrifuged at 1,500x*g* (4°C, 30 min). The resulting supernatant was stored at –80 °C until required. The concentration of protein in all samples was determined using the method of Bradford (1976).

3.2.3 Neurotransmitter receptor binding assays

For all receptor binding assays, 30 µg of membrane preparation was re-suspended in 100 µL of buffer (see Table 3.1 for specific details) and added to 96-well microplates containing a 1.0 µM GF/B glass filter (Millipore, Boston, MA, USA) according to established methods (Basu et al 2009). Prior to the addition of radioligands, samples were pre-incubated with plant extracts (final concentration = 0.5 mg/ml) for 30 min. All assay reactions were carried out under gentle agitation and terminated by vacuum filtration. The filters were rinsed three times with buffer and then allowed to soak for 96 hrs in 25 µL of OptiPhase Supermix Cocktail (Perkin Elmer, Waltham, MA, USA). Radioactivity retained by the filter was quantified by a liquid scintillation counter (Wallac Microbeta, Perkin Elmer, Waltham, MA, USA). Specific binding to the receptors was defined as the difference in radioligand bound in the presence and absence of 100 µM unlabelled displacer (Table 3.1). Percentage inhibition (expressed as "% of control binding") was determined by calculating specific binding in the presence and absence of extract. All samples were assayed in triplicate. Each assay run plate included test samples, as well as negative (blanks and solvent) and positive (displacer) controls.

3.2.4 Neurotransmitter enzyme assays

The neurotransmitter enzyme assays were used to determine the presence of potential compounds that may affect the activity of enzymes that have particular importance in the neuroendocrine reproductive axis in fish. The activities of AChE and MAO were measured using 96-well microplates according to published methods (Basu et al 2007, 2009) that are briefly outlined here. For AChE activity, 0.5 µg of supernatant protein was re-suspended to a final volume of 200 µl of buffer with 100 µM amplex red (10-acetyl-3, 7-dihydroxyphenoxazine), 200 mU horseradish peroxidase, 20 mU choline oxidase, and 100 µM acetylcholine. For MAO activity, 5 µg of protein was re-suspended to a final volume of 200 µl of buffer with 100 µM amplex red (10-acetyl-3, 7-dihydroxyphenoxazine), 200 mU horseradish peroxidase, and 100 mM tyramine. Following a 30 min incubation period for

both assays, the production of resorufin (λ_{ex} = 540, λ_{em} = 590) was monitored between 30 and 60 min (CytoFluor 2350, Millipore, Bedford, MA, USA). For GAD activity, 50 µg of brain homogenate was re-suspended in glass tubes to a final volume of 350 µl of 10 mM phosphate buffer (including 60 µM pyridoxal-5'-phosphate and 120 µM dithiothreitol, pH 7.4) according to published methods (Awad et al 2009). The reaction was initiated by addition of 30 mM glutamate containing 0.1 µCi l-[1-^{14}C] glutamic acid (60.0 mCi/mmol; Amersham, Buckinghamshire, England) and a suspended Whatman GF/B filter paper soaked in Scintigest (Fisher Scientific, Ottawa, Canada). Following a 45 min reaction at 37 °C, the reaction was terminated by the addition of 0.5 ml of 0.25 M HCl. The vials were allowed to incubate for another 60 min and the radioactivity trapped on the filter paper was determined.

For GABA-T activity, 300 µg of protein was re-suspended and incubated in 200 µl of 100 mM potassium pyrophosphate buffer (containing 5 mM α-ketoglutarate, 4 mM NAD, 3.5 mM 2-mercaptoethanol, 10 µM pyridoxal-5'-phosphate, pH 8.6) for 15 min at 37 °C according to published methods (Awad et al 2009). Following the addition of 10 mM GABA, absorbance was immediately monitored at 340 nM every 10 sec for 2 minutes and the maximal velocity (i.e., slope) of the enzymatic reaction (V_{max}) was calculated.

For all enzyme assays, samples were pre-incubated for 30 min with plant extracts at a final concentration of 0.5 mg/ml. Percentage inhibition (expressed as "% of control activity") was determined by calculating enzyme activity in the presence and absence of plant extract. The effect of solvent carrier was tested in each assay. All samples were assayed in triplicate.

3.2.5 Statistical Analyses

All statistical operations were performed with Sigma Stat (Version 2.03, SPSS Inc., San Rafael, CA, USA) using α = 0.05 as the level of statistical significance. The inhibitory effects of extracts (compared to non-exposed and solvent controls) were assessed by means of t-tests and ANOVAs (Tukey's post hoc, test for normality and Levene's test for homoscedasticity).

3.3 Results

3.3.1 Neurotransmitter receptor binding

Receptor binding assays showed species-specific activity for each receptor (Table 3.2). Butternut bark extract was the only significant inhibitor ($p < 0.05$) of muscimol binding to the GABA(A) receptor while the yellow birch bark extract was the only to significantly increase binding. Inhibitors of spiperone binding to the D2 receptor were red maple wood and bark, sugar maple bark, yellow birch bark, butternut wood, bitternut hickory wood, and yellow birch wood. In contrast shagbark hickory bark, beech bark, and largetooth aspen wood increased binding to the D2 receptor. Inhibitors of QNB binding to muscarinic ACh receptors were red maple wood and bark, ironwood wood, and hybrid poplar bark. Beech wood, basswood wood, shagbark hickory wood, green ash wood, largetooth aspen wood and bark, bitternut hickory bark, butternut bark, white birch wood, and sugar maple wood increased QNB binding to the muscarinic Ach receptor. Wood of elm, beech and ironwood increased NMDA receptor binding. There were no significant inhibitors of NMDA receptor binding found in this study.

3.3.2 Neurotransmitter enzyme activities

Enzyme inhibition was also species-specific (Figures 3.1 – 3.4). The AChE activity assay was sensitive to most extracts except elm bark, and wood of largetooth aspen and white birch. MAO activity was significantly inhibited by approximately half the extracts. GABA-T activity was selectively inhibited by red oak wood, white birch wood, hybrid poplar bark, basswood bark, bitternut hickory wood, beech bark, elm wood, shagbark hickory bark, ironwood wood, and largetooth aspen bark and was stimulated by yellow birch wood and red oak bark. GAD activity was inhibited by butternut bark and wood, elm wood, largetooth aspen wood, red maple bark, white birch wood, and yellow birch wood. GAD stimulation was observed by extracts of green ash bark, bitternut hickory wood, sugar maple wood, white birch bark, hybrid poplar bark, and ironwood wood.

Juglone (Fig. 3.5), a phytochemical present in butternut, showed a concentration-dependent activity against GAD (Fig. 3.6) with an IC_{50} of 3.35 μM (95% CI 2.47 – 4.55). The IC_{50} for MAO inhibition (data not shown) by juglone was 13.86 μM (95% CI 5.84 – 32.87 μM).

Table 3.2. Summary of *in vitro* receptor binding results with tree extracts, expressed as a mean % of control. For a given neurochemical parameter, asterisks represent significant differences ($p < 0.05$; n=3) from controls.

Tissue	Common name	Scientific name	GABA(A)	D2	mAChR	NMDA
wood	Green ash	*Fraxinus pennsylvanica*	127.7	64.4	169.7*	113.2
wood	Basswood	*Tilia americana*	99.8	68.6	155.3*	63.9
wood	Beech	*Fagus grandifolia*	95.8	129.2	347.1*	181.3*
wood	Bitternut hickory	*Carya cordiformis*	99.8	56.9*	128.1	139.8
wood	Butternut	*Juglans cinerea*	83.8	30.4*	135.8	142.5
wood	Elm	*Ulmus americana*	49.9	57.2	128.8	211.6*
wood	Ironwood	*Ostrya virginiana*	56.0	70.4	43.4*	185.2*
wood	Largetooth aspen	*Populus grandidentata*	90.7	165.0*	165.0*	142.5
wood	Red maple	*Acer rubrum*	76.0	6.3*	3.1*	104.3
wood	Red oak	*Quercus rubrum*	24.0	97.9	91.4	60.9
wood	Shagbark hickory	*Carya ovata*	99.8	106.9	176.7*	46.4
wood	Sugar maple	*Acer saccharum*	72.0	141.7	213.5*	66.1
wood	White birch	*Betula papyrifera*	98.7	74.1	182.9*	90.8
wood	Yellow birch	*Betula allegheniensis*	83.8	55.7*	108.1	107.3
bark	Green Ash	*Fraxinus pennsylvanica*	129.7	90.0	109.9	64.5
bark	Basswood	*Tilia americana*	149.7	102.1	120.0	105.0
bark	Beech	*Fagus grandifolia*	40.0	160.5*	97.0	73.1
bark	Bitternut hickory	*Carya cordiformis*	88.0	74.1	225.3*	49.5
bark	Butternut	*Juglans cinerea*	8.0*	78.7	187.3*	92.5
bark	Elm	*Ulmus americana*	73.3	105.5	58.6	60.6
bark	Hybrid poplar	*Populus sp*	62.7	108.8	19.4*	62.1
bark	Largetooth aspen	*Populus grandidentata*	139.7	123.7	151.6*	48.1
bark	Red maple	*Acer rubrum*	65.3	8.6*	45.3*	66.8
bark	Red oak	*Quercus rubrum*	37.9	56.6	67.1	52.3
bark	Shagbark hickory	*Carya ovata*	46.7	199.8*	98.5	94.4
bark	Sugar maple	*Acer saccharum*	89.8	18.6*	104.1	31.0
bark	White birch	*Betula papyrifera*	87.8	106.5	82.6	47.1
bark	Yellow birch	*Betula allegheniensis*	239.5*	21.1*	58.1	23.4

GABA(A), g-aminobutyric acid type A receptor; D2, dopamine type 2 receptor; mAChR, muscarinic acetylcholine receptor; NMDA, N-methyl-D-aspartate receptor.

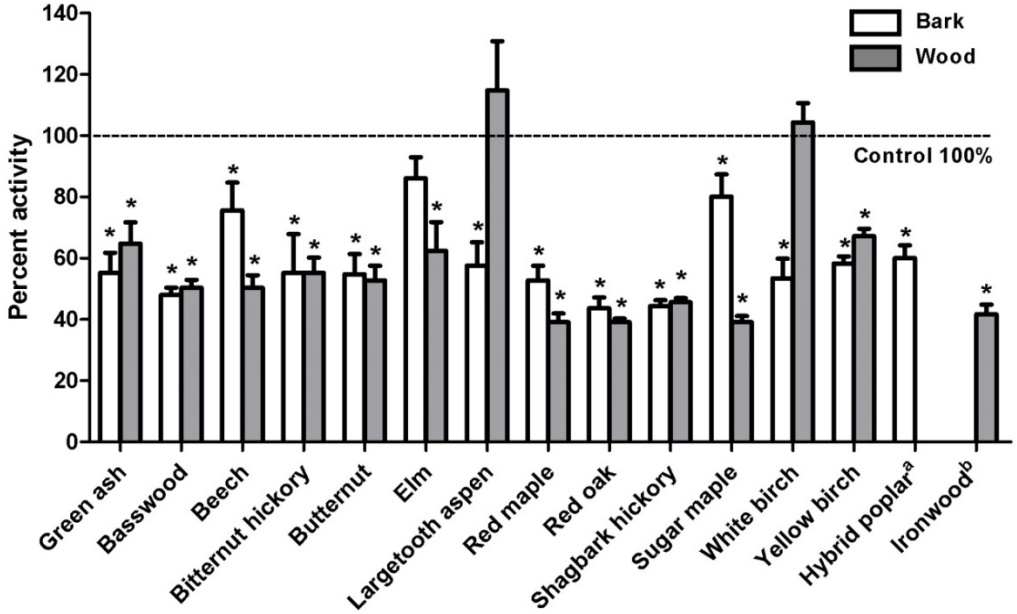


Figure 3.1 - Effects of hardwood bark and wood extracts on in vitro AChE activity expressed as percent activity of solvent controls. Extracts tested in triplicate at a final concentration of 0.5 mg/ml. Asterisk denotes $p < 0.05$. Solid line denotes control activity (100%). [a]Bark tested only. [b]Wood tested only.

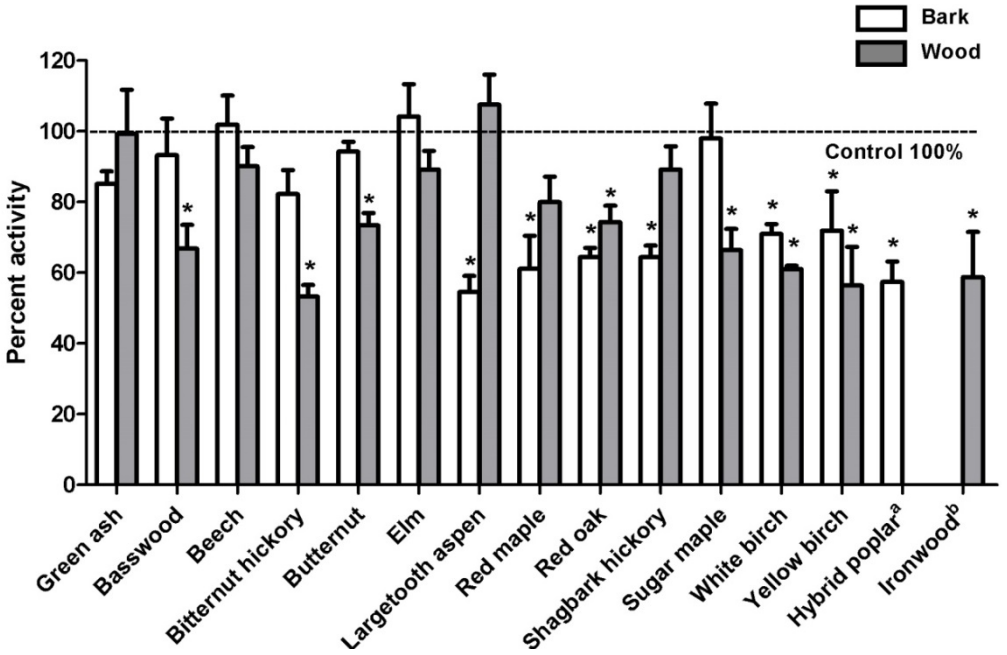


Figure 3.2 - Effects of hardwood bark and wood extracts on in vitro MAO activity expressed as percent activity of solvent controls. Extracts tested in triplicate at a final concentration of 0.5 mg/ml. Asterisk denotes $p < 0.05$. Solid line denotes control activity (100%). [a]Bark tested only. [b]Wood tested only.

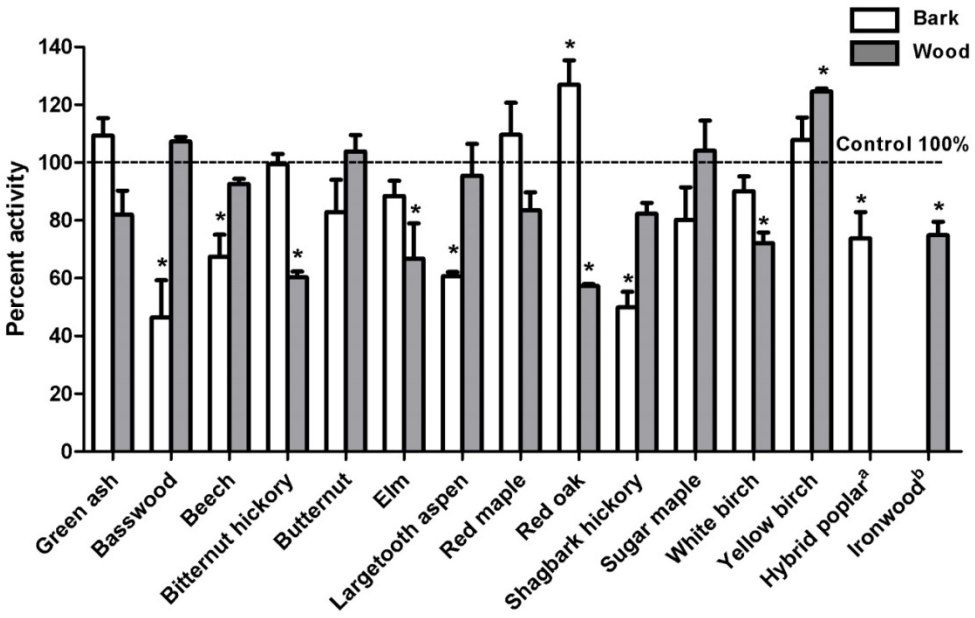


Figure 3.3 - Effects of hardwood bark and wood extracts on in vitro GABA-T activity expressed as percent activity of solvent controls. Extracts tested in triplicate at a final concentration of 0.5 mg/ml. Asterisk denotes $p < 0.05$. Solid line denotes control activity (100%). [a]Bark tested only. [b]Wood tested only.

3.4 Discussion

The key finding of this study was that extractable components in hardwood bark and wood contain neuroactive substances that, *in vitro*, directly interact with several neurotransmitter receptors and enzymes in the fish brain, demonstrating that the putative neuroactive compounds in pulp and paper mill effluents identified in my previous work (Basu et al 2009) may originate in the trees used as feedstocks.

The neuroactivities in pulp feedstocks may potentially have significant effects on fish reproduction downstream from mills. Pulp mill effluents are capable of rapidly and reversibly inhibiting spawning in the fathead minnow (Kovacs et al 2007). This indicates that effluent may be acting through disruption of neuroendocrine processes, as ovulation and sperm release are triggered by a rapid neuroendocrine cascade. One early study by Van Der Kraak et al (1992) demonstrated that in a white sucker (*Catostomus commersonii*) population downstream from a bleached Kraft mill, luteinizing hormone (LH) levels were suppressed and pituitary LH responsiveness to gonadotropin-releasing hormone (GnRH) was reduced. In a previous study I published with Basu et al (2009) and in Chapter 5 of this

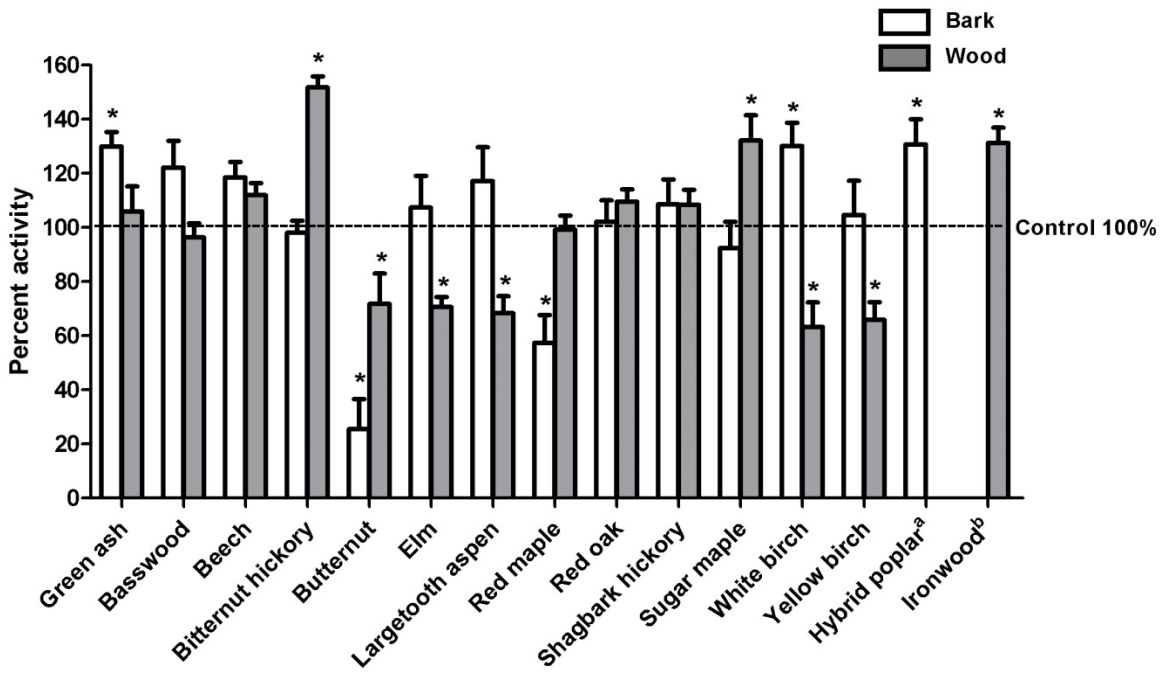


Figure 3.4 - Effects of hardwood bark and wood extracts on in vitro GAD activity expressed as percent activity of solvent controls. Extracts tested in triplicate at a final concentration of 0.5 mg/ml. Asterisk denotes $p < 0.05$. Solid line denotes control activity (100%). [a]Bark tested only. [b]Wood tested only.

thesis, I explored the potential for effluent to interact with key reproductive neuroendocrine mechanisms, and this paper extends the neuroendocrine disruption hypothesis (Waye and Trudeau 2011) to include the idea that active principles originate in pulp and paper feedstocks.

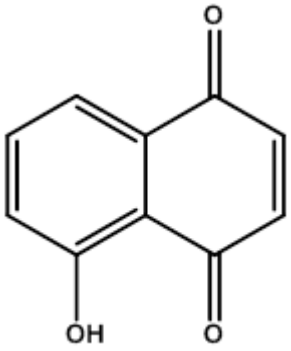


Figure 3.5 - Juglone, a phytochemical present in butternut tree.

Dopamine is a potent inhibitor of GnRH and LH release in multiple teleost species (Dufour et al 2010, Peter et al 1986) and GABA is demonstrated to be a key stimulator of GnRH and LH release in goldfish (Trudeau 1997, Popesku et al 2008) with the glutamatergic and cholinergic systems also stimulating GnRH release (Trudeau 1997, Krsmanovic et al 1998). My findings that compounds in hardwood feedstocks can selectively modulate and bind to key receptors in the brain that control reproduction are somewhat difficult to interpret. For example,

binding to the receptor in these assays did not elucidate if the agent is a chemical agonist or antagonist, but it clearly demonstrates that neurotransmitter disruption can occur. Notably, approximately half of the extracts interacted with the dopamine receptor that plays a powerful role in the inhibition of spawning. This inhibitory action is potent, and it is necessary to co-inject specific D2 receptor antagonists along with GnRH agonists to induce LH release and ovulation in many fish species. To elucidate if the ligands present in my samples are agonists or antagonists, additional mechanistic studies will be required.

The effects of extracts on enzymes which synthesize or metabolize neurochemicals are more easily interpreted, as I can infer how these effects may impact the reproductive axis

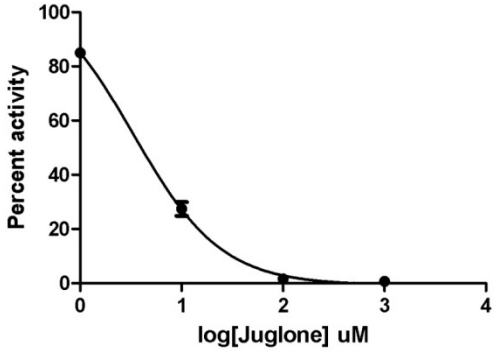


Figure 3.6 - Effect of juglone from butternut on in vitro GAD activity expressed as percent activity of solvent controls. IC50 = 3.35 µM (r^2 > 0.995, 95% C.I. = 2.47 – 4.55, n = 3).

if effects do indeed occur *in vivo*. MAO is an important enzyme in the metabolism of dopamine and half of the tested extracts inhibited this enzyme. With less MAO activity, I would expect levels of dopamine in the brain to increase, thereby inhibiting GnRH and LH release. Inhibitions in the enzyme GAD would also be detrimental to reproduction. GAD synthesizes GABA from glutamate, and with decreased levels of GABA, I would expect a loss in the stimulatory pathway for GnRH and LH release. Seven of the tested extracts inhibited GAD. GABA-T, the enzyme that metabolizes GABA, was stimulated by two of the extracts. With increased GABA metabolism, I would also expect less GABA to be present to stimulate the reproductive axis. AChE was inhibited by most samples and the potential increase of ACh could stimulate GnRH release.

Due to the important roles of dopamine and GABA, as strong inhibitors and stimulators respectfully, in the reproductive axis, I was particularly interested in GAD and MAO enzyme inhibition. In this study, butternut wood significantly inhibited both of these

enzymes (Fig. 3.2 and 3.4), and butternut bark inhibited GAD activity (Fig. 3.4). I also had the pure compound juglone, a major secondary metabolite from butternut and I determined IC_{50} values of 3.35 μM for GAD (Fig. 3.5) and 13.86 μM (95% CI 5.84 – 32.87 μM) for MAO.

Clearly there is considerable scope for bioassay-guided isolation of active principles from the many extracts identified as inhibitory to GAD and MAO. Any hardwood phytochemicals that are found to be inhibitory to these enzymes can be tested in *in vivo* models. Subsequent targeted identification and testing of active compounds in mill effluents and in the tissues of exposed fish where hardwoods are used as feedstocks would also be possible. Based on my *in vitro* findings in this study, priority samples for bioassay-guided isolation are: butternut, as the wood and bark inhibit GAD and the wood inhibits MAO; white and yellow birch wood, which inhibit both GAD and MAO; and red maple bark, which also inhibits these two enzymes.

To be biologically active in fish or other aquatic species, phytochemicals would have to survive the temperature, pressure, and chemical changes of the pulping and effluent treating (i.e., primary and secondary treatment) processes, be bioavailable to exposed fish, and be distributed to the site of action, all of which remains to be demonstrated. Nevertheless, the identification of neuroactive substances in these extracts could lead to better monitoring of effluents for the presence of potential neuroendocrine disruptors at mills where these hardwoods are used.

These tested extracts and juglone also provide a wide range of neuropharmacological potential that may indicate new sources of natural health products as the pathways explored in this chapter are common drug targets. Though herbs and medicinal plants have been widely studied for their neuropharmacological properties, much less is known about the neuropharmacological potential of Canadian forest trees. These active hardwood extracts may be leads for medicinal products or for further study of the chemical ecology of plant-herbivore interactions. In particular, the presence of GABAergic phytochemicals could lead to the identification of new plant extracts and compounds with anxiolytic activity. For example, GABAergic modulators have already been found in anxiolytic medicinal plants such as skullcap *Scutellaria lateriflora* (Awad et al 2003) and sin susto, *Souroubea*

sympetala (Mullally et al 2011), which contain the bioactive GABA(A) benzodiazepine binding site agents scutellarin and betulinic acid, respectively. GABA-T is another GABAergic pharmacological target for calming activity (or epilepsy) and is inhibited by rosmarinic acid and other phytochemicals in lemon balm (Awad et al 2009). Foods and medicinal products containing MOA inhibitors such as harman and quercetin may be useful in treating depression (Dixon Clarke and Ramsey 2010). Changes of NMDA binding activity in the cerebellum of rats induced by the neurotoxin methyl-mercury can be restored by phenolic rich extracts of Labrador tea (*Rhododendron tomentosum* ssp. subarcticum) (Black et al 2011). Galanthamine, from snowdrop (*Galanthus sp.*), is a mild AChE inhibitor and is useful in treatment of Alzheimer's disease (Koda et al 2011). Clearly there is potential for identification of other secondary metabolites with interesting biological activities from many of these extracts, unlocking potential growth in the Canadian forest product industry in addition to increasing the understanding of the ecological ramifications (i.e., neuroendocrine disruption) that mill effluents may pose to exposed fish.

3.5 Conclusion

The research in this chapter clearly demonstrates that neuroactive compounds exist in Canadian forest hardwood trees used for pulp and paper production, and that juglone, a phytochemical in butternut, has demonstrable MAO and GAD inhibitory activity. These findings can help guide research towards the identification of neuroendocrine disruptors at pulp mills where these hardwood feedstocks are being used as well as provide an avenue of research for the development of natural health products for the treatment of neurological disorders affected by the neurotransmitter systems explored.

Chapter 4: Canadian boreal pulp and paper feedstocks contain neuroactive substances that interact *in vitro* with GABA and dopaminergic systems in the brain

[1]Andrew Waye, [1]Malar Annal, [1]Andrew Tang, [1]Gabriel Picard, [1]Frédéric Harnois, [1]Jose-Antonio Guerrero-Analco, [1]Ammar Saleem, [2]L Mark Hewitt, [2]Craig B Milestone, [3]Deborah L MacLatchy, [1]Vance L Trudeau, [1]John T Arnason

[1]Department of Biology, University of Ottawa, Ottawa, ON, K1N 6N5

[2]Canadian Centre for Inland Waters, Environment Canada, Burlington, ON, L7S 1A1

[3]Department of Biology, Wilfred Laurier University, Waterloo, ON, N2L 3C5

Following the identification of neuroactivities in extracts of hardwood feedstocks of pulp and paper mills, this study examines the neuroactivities of conifer feedstocks more commonly used in Canada. Phytochemical standards associated with pulp mill effluents were also acquired and tested. In this chapter, a more thorough interdisciplinary examination of my results on the potential for natural health product development is performed. This study has been published in Science of the Total Environment (Waye et al 2014a) with minor modifications for this thesis.

Statement of author contributions

AW, VLT, and JTA conceived and designed this study. AW, JAGA, and AS prepared extracts, and MA, AT, GP, and AW performed bioassays. Phytochemicals were provided by LMH, CBM, and DLM. Literature review of phytochemicals for Table 4.2 performed by BH. AW performed data analysis and prepared the manuscript with contributions from JAGA, AS, LMH, CBM, DLM, VLT, and FTA.

4.1 Introduction

Conifer forests have long been used in the pulp and paper mill industry as a source of cellulose fibre. Following cellulose extraction, effluents generated from this industry are rich in plant secondary metabolites which, after treatment, are released to aquatic ecosystems. In the research chapters of this thesis and publications with collaborators, I have shown that these by-products from wood processing demonstrated the potential to interfere with normal neuroendocrine processes in exposed fish populations (Basu et al 2009, Milestone et al 2012, Waye and Trudeau 2011).

Reproductive and metabolic disruption of wild fish downstream of pulp and paper mills (resulting in smaller gonads, larger livers, later age to maturity, and increased condition factor) have been an important research focus for environmental toxicologists and endocrinologists for the last decade and a half (for reviews, see Hewitt et al 2006, McMaster et al 2006, Parrot et al 2006) with concerted efforts towards solutions (Hewitt et al 2008). Furthermore, fathead minnows exposed to effluents in laboratory studies exhibit a rapid and reversible inhibition of spawning (Kovacs et al 2007). Disruption at the level of the brain has been an overlooked mechanism involved in these perturbations as compounds in effluent may interfere with hypothalamic neurotransmitter and neuropeptide pathways critical for the regulation of pituitary and gonadal function (Basu et al 2009, Popesku et al 2010). This has led to the neuroendocrine disruption hypothesis for the actions of pulp and paper mill effluents to inhibit reproductive processes in fish (Basu et al 2009, Waye et al 2011).

In vitro screening studies in Chapter 2 and publications with collaborators (Basu et al 2009, 2012, Milestone et al 2012) have shown that effluents and wood feedstock extracts are highly active at enzyme and receptor sites in the neuroendocrine γ-aminobutyric (GABA) and dopamine signaling pathways which tightly regulate the reproductive axis (Popesku et al 2008). Prior to this work, very few studies had been undertaken to assess the impact of pulp mill effluents on the fish brain. One study by Van Der Kraak et al. (1992) demonstrated the effects of effluents on pituitary function, where luteinizing hormone (LH) levels were lower

in populations of white sucker (*Catostomus commersonii*) downstream of mill discharge. When these exposed fish were injected with a gonadotropin-releasing hormone (GnRH) agonist, the LH surge and subsequent ovulation seen in control fish was not observed.

I have previously reported on the different neuroendocrine bioactivities of extracts from Canadian hardwood feedstocks (Basu et al 2012)(Chapter 2) and mixed feedstocks used in Brazil, New Zealand, and Canada (Milestone et al 2012), however there is a lack of information on the most commonly pulped and phytochemically distinct boreal conifer species. Therefore the objective of the present study was to screen the neuroendocrine activities of extracts from nine boreal conifer species and sixteen conifer-derived phytochemicals against a battery of *in vitro* assays focussing on activities in the dopamine and GABA neurotransmitter systems. It is important to note that these phytochemicals have been identified in final mill effluents (for example, resin acids) or detected in Canadian softwood Kraft mill chemical recovery condensates associated with hormonal disruptions (Belknap et al 2006). Furthermore, the neurotransmitter systems I explore have well-documented and critical roles in reproductive neuroendocrine control mechanisms (Basu et al 2009, Popesku et al 2008, Trudeau 1997) and numerous neurological ailments from anxiety or epilepsy (Awad et al 2007, 2009, Mullally et al 2011) to Parkinson's disease and its associated disorders (Weintraub and Burn 2011).

I measured the enzymatic activities of monoamine oxidase (MAO), glutamic acid decarboxylase (GAD), and GABA transaminase (GABA-T), and binding to the GABA(A) receptor benzodiazepine binding site (GABA(A)-BZD). I chose these assays in response to the fact that in vertebrates, GABA and dopamine are among the most abundant neurotransmitters and play important roles in diverse behavioural processes while controlling the endocrine axes.

MAO is one of the enzymes responsible for metabolizing the neurotransmitters dopamine, serotonin, and norepinephrine, thereby inactivating them. There are two sub-types of MAO in mammals (MAO-A and MAO-B) which have differing substrate selectivity and inhibitor sensitivity. In goldfish (*Carassius auratus*), there is only one form of MAO, corresponding more closely to the mammalian MAO-A; that is, it is more sensitive to MAO-A type inhibitors

(Figueroa et al 1981). Dopamine is the most important inhibitor of the reproductive axis in fish, inhibiting release of GnRH in the hypothalamus and LH from the pituitary. GAD is the enzyme responsible for synthesizing the neurotransmitter GABA while GABA-T is responsible for its metabolism. There are three receptors for GABA. The GABA(A) receptor is important for stimulating GnRH in the hypothalamus. GnRH in turn stimulates LH release from the pituitary which causes ovulation in females, sperm release in males, and sex steroid production in both sexes (Popesku et al 2008, Trudeau 1997). When an agonist binds to GABA(A)-BZD, the activation of the GABA(A) receptor by GABA is potentiated (Smith and Olsen 1995).

I tested the inhibition of MAO and GAD enzyme activities in goldfish brain extracts with the following objectives: 1) to determine the potential of these chemicals to disrupt neuroendocrine functions related to reproduction in fish exposed to pulp mill effluents, and 2) to determine the pharmacological potential of conifer extracts and their secondary metabolites in vertebrates. To further explore the pharmacological potential of conifers and their phytochemicals, I included the GABA-T enzyme activity and GABA(A)-BZD receptor-binding assays that are currently established in our lab and optimized with brain preparations from rats (*Rattus norvegicus*). These two assays, as well as the GAD assay, have previously been used to examine the effects on the GABAergic system of plants traditionally used by the Q'eqchi Maya in the treatment of epilepsy and anxiety (Awad et al 2007, 2009). The GABAergic and dopaminergic systems are important targets for the pharmaceutical treatment of many neurological disorders ranging from Parkinson's disease (such as the MAO inhibitor rasagiline) to anxiety (GABA(A)-BZD agonists such as diazepam) and epilepsy (GABA-T inhibitors such as vigabatrin). The pharmaceutical treatments for these disorders, such as diazepam or vigabatrin, can have serious side effects with prolonged usage; therefore, the identification of alternatives was an important avenue to consider during my exploration of the neuroendocrine disruption of reproduction due to the common mechanisms of action.

4.2 Methods

4.2.1 Plant collection

Common juniper (*Juniperus communis* L.), white spruce (*Picea glauca* (Moench) Voss), black spruce (*Picea mariana* (Mill.) BSP.), balsam fir (*Abies balsamea* (L.) Mill.), and jack pine (*Pinus banksiana* Lamb.) were collected in September 2007 near Mistissini, QC, Canada. White cedar (*Thuja occidentalis* L.), eastern hemlock (*Tsuga canadensis* (L.) Carr.), tamarack larch (*Larix laricina* (Du Roi) Koch), and white pine (*Pinus strobus* L.) were collected near Denholm, QC, Canada, in 2008. Plants were classified according to Scoggan (1978). Voucher numbers are shown in Table 4.1 and are deposited at the herbarium of the University of Ottawa (Ottawa, ON, Canada).

Table 4.1. List of Eastern Canadian conifer species tested, with Latin and common names and voucher numbers for the herbarium at the University of Ottawa. Specimens were classified according to Scoggan (1978).

Plant	Voucher no.
Abies balsamea L. (Balsam fir)	19980
Picea mariana (Mill.) BSP (Black spruce)	19981
Juniperus communis L. (Common juniper)	19982
Picea glauca (Moench) Voss (White spruce)	19983
Larix larcina (Du Roi) Koch (Tamarack larch)	19984
Pinus banksiana Lamb. (Jack pine)	19985
Tsuga canadensis (L.) Carr. (Eastern hemlock)	19986
Thuja occidentalis L. (White cedar)	19987
Pinus strobus L. (White pine)	19988

4.2.2 Phytochemical standards

Standards for the resin acids neoabietic acid, abietic acid, levopimaric acid, palustric acid, sandaracopimaric acid, and isopimaric acid were provided by FPInnovations (Pointe-Claire, QC, Canada), while dehydroabietic acid, geranyl linalool, 4-ethylguaiacol, vanillin, veratraldehyde, manool, isoeugenol, 3-hydroxy-5-methoxystilbene, and pinosylvin, described in Belknap et al (2006) and Parrott et al (2011), were provided by Environment Canada's Centre for Inland Waters (Burlington, ON, Canada).

4.2.3 Preparation of plant extracts

The tree samples were debarked and chipped by hand using a sharp blade. The chips were dried in an oven overnight at 40 °C before being ground to sawdust using a Thomas-Wiley Laboratory Mill Model 4 (Arthur C. Thomas Company, Philadelphia, PA, USA) with a 2 mm mesh screen. Two sawdust samples from each species were extracted; one with methanol, and the other with ethylacetate (ACS grade, Fisher Scientific, Ottawa, ON, Canada). The extraction was performed for 48 hours with a ratio of 50 ml of solvent per gram of dried plant material. Prior to sample preparation, I used a classic polarity series for exploratory solvent extractions with two of the tree samples. Non-polar hexane extracts of wood were prepared, but the yields were inadequate for testing. The mid-polar ethylacetate extraction captured most of the non-polar phytochemicals seen in the hexane extract as observed using thin layer chromatography. The methanol extract contained more polar substances with only slight overlap with the ethylacetate extract. Dichloromethane (DCM), 80% ethanol, and 1:1 DCM:methanol extractions all yielded extracts with similar phytochemical profiles as the ethylacetate and methanol extracts as determined by TLC. After extraction, samples were vacuum filtered using Whatman 1 filter paper (pore size 11 µm, diameter 11 cm, Fisher Scientific, Ottawa, ON, Canada) and rotoevaporated to dryness at 40 °C at 950 mmHg. Samples were stored in glass in the dark at 4 °C until tested. All extracts were dissolved at 5 mg/ml in ACS grade DMSO (Fisher Scientific, Ottawa, ON, Canada) prior to testing.

3.2.4 Preparation of goldfish and rat brain tissues

For collection of goldfish brains, male and female goldfish (*Carassius auratus*) were purchased from Aleong's International Inc. (Mississauga, ON, Canada) and allowed to acclimate in the University of Ottawa Aquatic Care Facility for two months in 55 L tanks at 18 °C in dechlorinated City of Ottawa tap water. Fish were fed twice daily ad libitum and under a natural simulated photoperiod (fish were sampled in September). The fish were anesthetized with MS-222 (Syndel Laboratories, Qualicum, BC, Canada), decapitated, and whole brains were dissected, combined, and stored at -80 °C until used. For collection of rat tissues, male Sprague-Dawley rats (Charles River Laboratories Inc., St. Constant, Quebec) that were housed individually and maintained under standard animal room conditions (clear plexiglass cages, 24 x 30 x 18 cm, 12 h light-dark cycle, 21±1 °C, 60% humidity, Purina Lab

Chow and tap water ad libitum) were anaesthetized with CO_2/O_2, sacrificed, and whole brains were dissected, flash frozen in liquid nitrogen, and stored at -80 °C until used. All experimental procedures were approved by the Research Ethics Committee of the University of Ottawa and met the guidelines set out by the Canadian Council on Animal Care (CCAC).

In vitro enzyme assays were performed according to previously validated methods (Basu et al 2009). For MAO activity, whole goldfish brain tissue was sonicated immediately after dissection in cold Na/K buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4) with 0.5% (v/v) Triton X-100 and then centrifuged at 15,000 × *g* (4 °C, 10 min). Supernatant was removed and stored in 0.5 ml aliquots at -80 °C. For the GAD enzyme activity assay, whole goldfish brains were homogenized in 1:10 (w/v) 10 mM phosphate buffer solution (pH 7.5) and stored in 1 ml aliquots at -80 °C until used. For GABA-T enzyme activity, whole rat brains were homogenized in 1:10 (w/v) buffer (20% glycerol, 0.13% Triton X-100, 0.1 mM reduced glutathione, 0.1 mM pyridoxal-5'-phosphate, 1 mM Na_2EDTA, 10 mM K_2HPO_4, pH 6.8) and centrifuged at 1,500 × *g* (4 °C, 30 min). The supernatant was stored at -80 °C until required.

The GABA(A)-BZD receptor binding assay tissues were prepared according to previously published methods (Awad et al 2009). Whole rat brains were homogenized in 1:10 (w/v) in buffer (50 mM Tris HCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$), centrifuged at 32,000 × *g* (4 ºC, 15 min) and the resulting pellet was washed twice under the same conditions. The concentration of protein in all samples was determined using the method of Bradford (1976).

4.2.5 Neurochemical enzyme and GABA(A)-BZD receptor binding activities

Monoamine oxidase activity was measured in quadruplicate, using the Amplex Red Monoamine Oxidase Assay Kit (cat. #A12214, Invitrogen, Burlington, ON, Canada). Extracts were tested at a final concentration of 5 µg/ml and pure compounds were tested at 100 µM with 20 µg of brain protein. The MAO inhibitor clorgyline (1 µM) was used as a positive control for MAO inhibition. The production of resorufin was monitored (λ_{ex} = 544, λ_{em} = 590) for 2 hours after reaction initiation using a SpectroMax M5 (Molecular Devices, Sunnyvale, CA, USA).

Glutamic acid decarboxylase activity was measured in triplicate by adding 50 µg of brain protein to glass tubes containing extracts at a final concentration of 50 µg/ml and pure

compounds at 1 mM. 3-mercaptopropionic acid (3-MPA; 1 mM) was used as a positive control for GAD inhibition. Generated radioactive CO_2 trapped on the filter paper was measured using a LS6500 Multipurpose Scintillation Counter (Beckman Coulter, Mississauga, ON, Canada).

For GABA-T activity, 300 µg of protein was incubated in triplicate for 15 minutes at 37°C with the final concentration of tested samples at 50 µg/ml for extracts or 1 mM for pure compounds. After incubation, 10 µM of GABA was added to the reactions and absorbance was monitored at 340 nM every 10 sec for 2 minutes and the maximal velocity of the enzymatic reaction (V_{max}) was calculated. Gamma-vinyl GABA (GVG; 1 mM), a pharmaceutical antiepileptic, was used as a positive control for GABA-T inhibition.

For all enzyme assays, percentage inhibition (expressed as "% of control activity") was determined by calculating enzyme activity in the presence and absence of sample. The effect of the solvent carrier and a positive control were tested in each assay.

For the GABA(A)-BZD receptor binding assay, 30 µg of rat brain protein was incubated in triplicate with extracts at 50 µg/ml or pure compounds at 1 mM. Radioactivity was measured with a Wallac MicroBeta TriLux microplate scintillation counter (PerkinElmer, MA, USA). Displacement (expressed as a "% of control binding") was determined by calculating displacement of radioligand in the presence and absence (solvent carrier only) of sample.

Statistics were performed with SPSS Statistics 17.0 (IBM Corporation, Somers, NY, USA) using one-way ANOVA with a Tukey's post-hoc analysis, normality, and Levene's test. For data without homogeneity of variance, Dunnett's T3 post-hoc analyses were performed. All significant results were determined at $p < 0.05$.

4.3 Results

4.3.1 Monoamine oxidase activities

In this study, goldfish MAO was significantly inhibited *in vitro* by all conifer extracts except the ethylacetate extract of white pine. The methanol extracts of white and black spruce were the most active (57% and 56% inhibition respectively; Fig. 4.1) while the least active methanol extracts were white pine (23% inhibition) and jack pine (27% inhibition). White spruce was also the most active ethylacetate extract, causing 59% inhibition of MAO

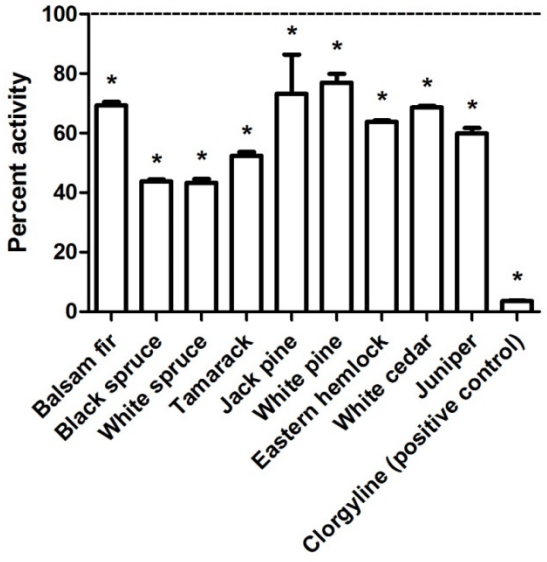


Figure 4.1 - Effects of conifer methanol extracts on in vitro MAO activity expressed as mean percent activity of solvent controls. Error bars represent one standard error. Extracts tested in quadruplicate with final concentration of 5 µg/ml. Asterisk denotes $p < 0.05$. Dotted line denotes control activity (100%).

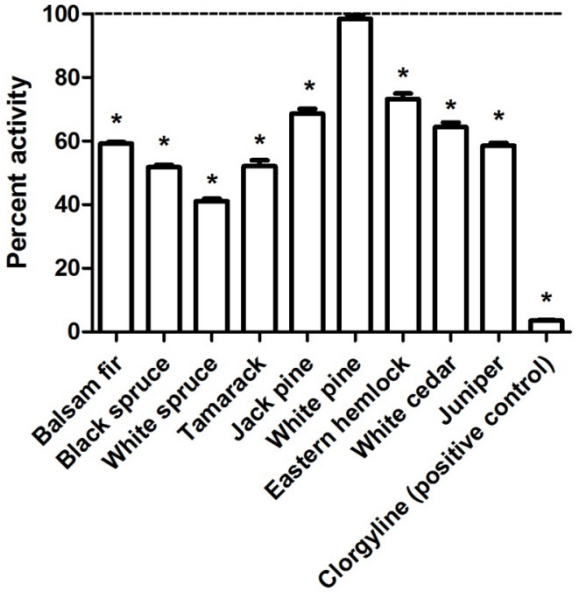


Figure 4.2 - Effects of conifer ethylacetate extracts on in vitro MAO activity expressed as mean percent activity of solvent controls. Error bars represent one standard error. Extracts tested in quadruplicate with final concentration of 5 µg/ml. Asterisk denotes $p < 0.05$. Dotted line denotes control activity (100%).

(Fig. 4.2). The other ethylacetate conifer extracts caused between ~30-50% inhibition with the exception of white pine, which was inactive.

Many of the pure compounds I tested were active (Fig. 4.3), with five significantly inhibiting MAO activity, such as isoeugenol (100% inhibition), 4-ethylguaiacol (57% inhibition), and vanillin (49% inhibition). Four were stimulatory, including the linear diterpene geranyl linalool (189% stimulation), the stilbene pinosylvin (28% stimulation) and

the resin acids levopimaric acid (56% stimulation) and palustric acid (22% stimulation). The remaining six compounds were not significantly active.

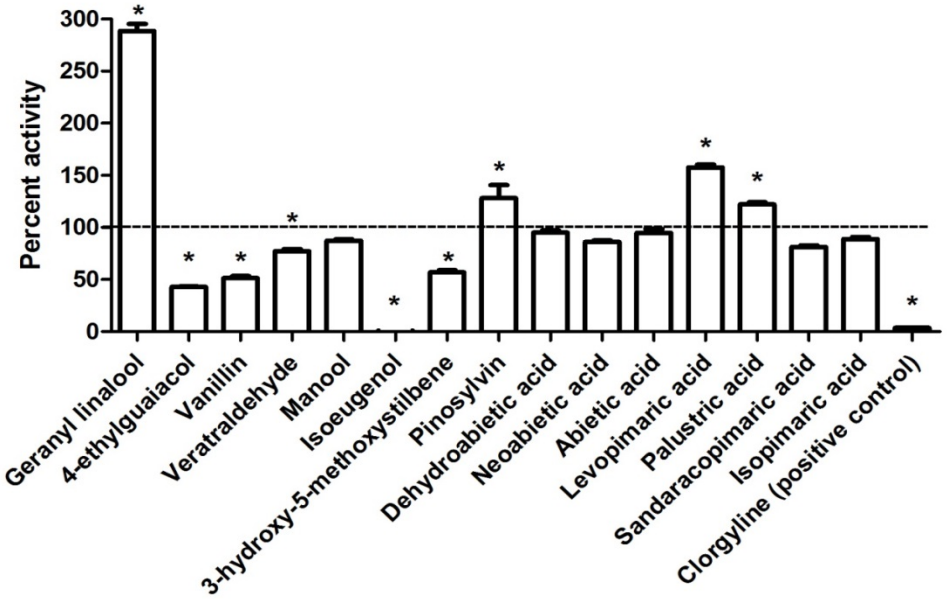


Figure 4.3 - Effects of phytochemical standards on in vitro MAO activity expressed as mean percent activity of solvent controls. Error bars represent one standard error. Extracts tested in quadruplicate with final concentration of 100 μM. Asterisk denotes $p < 0.05$. Dotted line denotes control activity (100%).

4.3.2 Glutamic acid decarboxylase activities

Conifer extracts were tested in the GAD enzyme assay using goldfish tissues and methanol extracts of balsam fir, white spruce, tamarack, jack and white pine, and white cedar were significantly inhibitory (Fig. 4.4). Of the active conifer methanol extracts, all had between 14-20% inhibition. Black spruce, eastern hemlock, and juniper were not significantly different from solvent controls. The balsam fir and white cedar ethylacetate extracts were the most inhibitory to GAD showing 30% inhibition (Fig. 4.5). Black and white spruce, tamarack, jack and white pine, and eastern hemlock were also active, with inhibition between 14-25%. Juniper was not statistically different from solvent controls. Ten of the sixteen tested phytochemicals were found to be inhibitory to GAD (Fig. 4.6). Of these, all of the resin acids (dehydroabietic acid 52% inhibition, neoabietic acid 56%, abietic acid 98%, levopimaric acid 53%, palustric acid 54%, pimaric acid 55%, sandaracopimaric acid

64%, isopimaric acid 55%) were among the most inhibitory. The stilbenes pinosylvin (43% inhibition) and 3-hydroxy-5-methoxystilbene (22% inhibition) also inhibited GAD.

4.3.3 GABA-transaminase activities

None of the conifer extracts nor any of the phytochemical standards were found to change rat GABA-T enzyme activity *in vitro* compared to controls.

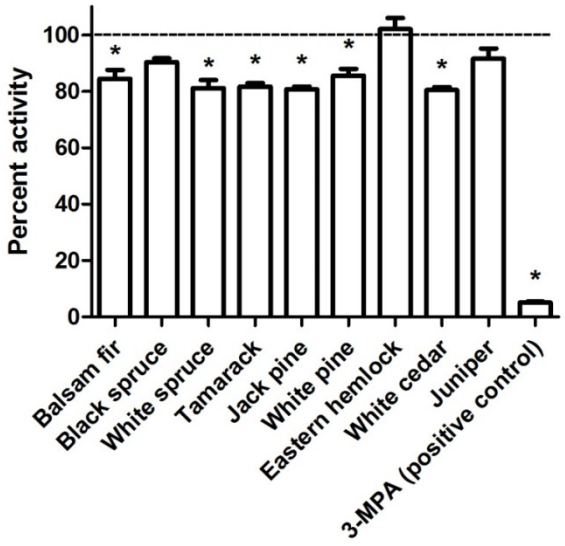


Figure 4.4 - Effects of conifer methanol extracts on in vitro GAD activity expressed as mean percent activity of solvent controls. Error bars represent one standard error. Extracts tested in triplicate with final concentration of 50 µg/ml. Asterisk denotes $p < 0.05$. Dotted line denotes control activity (100%).

4.3.4 GABA(A)-BZD binding activities

Only the methanol extract of juniper (94% displacement of ligand) and the ethylacetate extract of white spruce (58% displacement) were found to be significantly active in the GABA(A)-BZD assay performed using rat brain tissue. None of the tested pure compounds displaced flunitrazepam as GABA(A)-BZD ligands.

4.4 Discussion

In this study I showed the ability of conifer extracts to inhibit MAO and GAD and that specific phytochemicals found in these feedstocks were inhibitory to GAD, inhibitory or stimulatory to MAO, and were able to bind to the GABA-A receptor benzodiazepine binding site. In this study, I have reviewed the literature and compiled the presence of each of the phytochemicals in each of the conifers tested and the tissue in which they were found (Table 4.2). My bioassay results demonstrate a plausible mechanism for reproductive disruption in fish residing

below Canadian pulp mill outfalls where these boreal species are commercially processed and their phytochemicals may be released to the aquatic environment. The ability of conifer phytochemicals to be discharged in effluent is explored in Chapter 6. These results also provide a future direction for natural products research concerning neurological disorders where dopamine and GABA are important treatment targets.

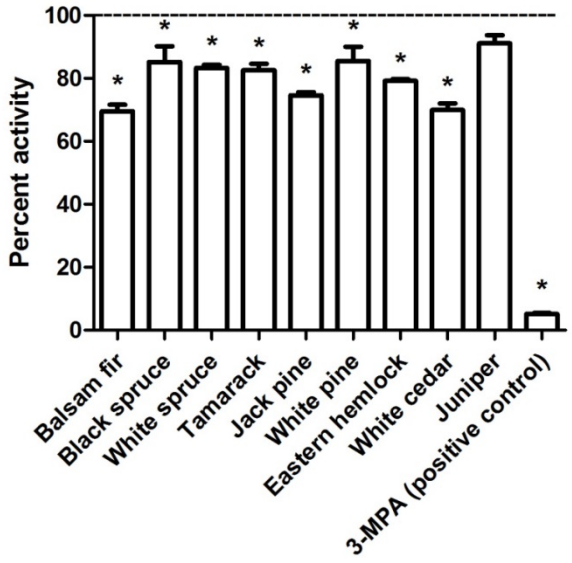


Figure 4.5 - Effects of conifer ethylacetate extracts on in vitro GAD activity expressed as mean percent activity of solvent controls. Error bars represent one standard error. Extracts tested in triplicate with final concentration of 50 µg/ml. Asterisk denotes $p < 0.05$. Dotted line denotes control activity (100%).

4.4.1 Monoamine oxidase

Inhibition of MAO by the majority of the methanol and ethylacetate extracts of these conifer pulp feedstocks, as well as strong inhibition by some phytochemicals such as isoeugenol, could result in an increase of dopamine in the brains of fish exposed to pulp mill effluents in the environment. This increase in dopamine could in turn suppress GnRH and LH. Dopamine is the primary inhibitor of GnRH and LH release in fish (Dufour et al 2010), being so potent it is necessary to co-inject dopamine receptor antagonists with GnRH to stimulate spawning in many fish species. In the studies I published with Basu et al (2009) and Milestone et al (2012), it was the most polar fractions of final pulp mill effluents that showed the greatest inhibition of MAO activity. In the current analysis of feedstock extracts, both the polar methanol and the less polar ethylacetate extracts had significant MAO inhibitory activity. Eastern hemlock, white pine, and black spruce had a more active methanol (polar) fraction, while balsam fir had a more active ethylacetate fraction.

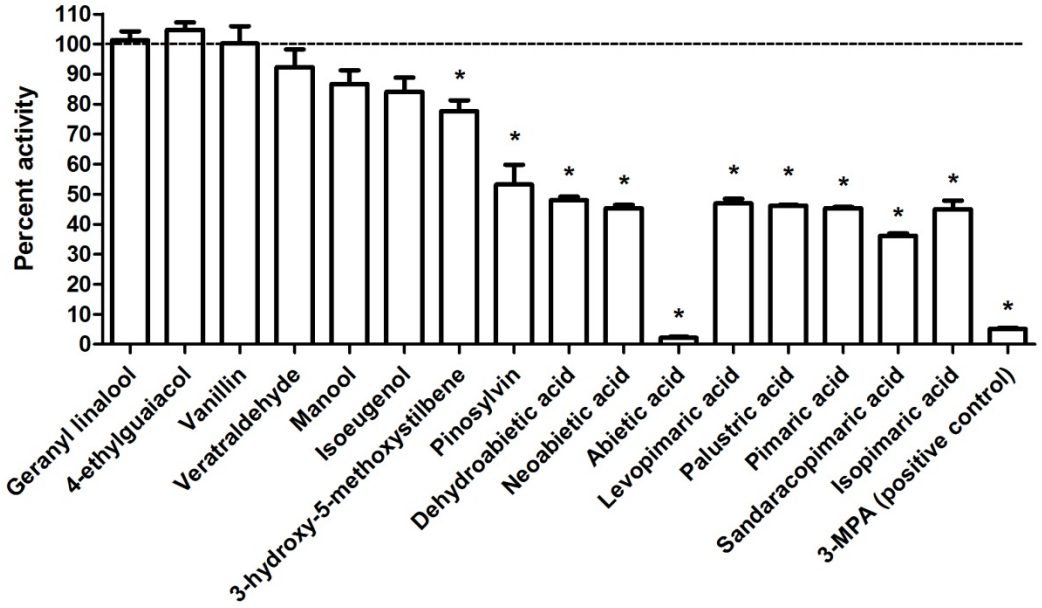


Figure 4.6 - Effects of phytochemical standards on in vitro GAD activity expressed as mean percent activity of solvent controls. Error bars represent one standard error. Extracts tested in triplicate with final concentration of 1 mM. Asterisk denotes $p < 0.05$. Dotted line denotes control activity (100%).

Inhibitors of MAO are important therapeutic agents for the treatment of anxiety and depression and can have neuroprotective actions for Alzheimer's or Parkinson's disease (Youdim 2010). My results indicate a structure-activity relationship for MAO inhibition (Fig. 4.7). Isoeugenol, vanillin, and 4-ethylguaiacol all share a similar structure with the only difference being substitutions para to the hydroxyl group on the benzene ring. Of these three phytochemicals, the 1-propylene substitution conferred the highest MAO inhibition, with the ethyl and aldehyde substitutions showing decreasing MAO inhibition. When comparing vanillin to veratraldehyde, I found that methylation of the hydroxyl group resulted in a decreased MAO inhibition, while the similarly structured 1,3,5 substituted 3-hydroxy-5-methoxystilbene shows an intermediate MAO inhibition. All of the MAO inhibitors identified in this study are commonly found in various conifers, including the wood used for pulp production (Table 4.2). Furthermore, this structure-function correlation identifies an avenue for the synthesis of related structures to be tested for MAO inhibition, perhaps

leading to the identification of potential new pharmaceutical MAO inhibitors. All of these phytochemicals share structural similarities to dopamine.

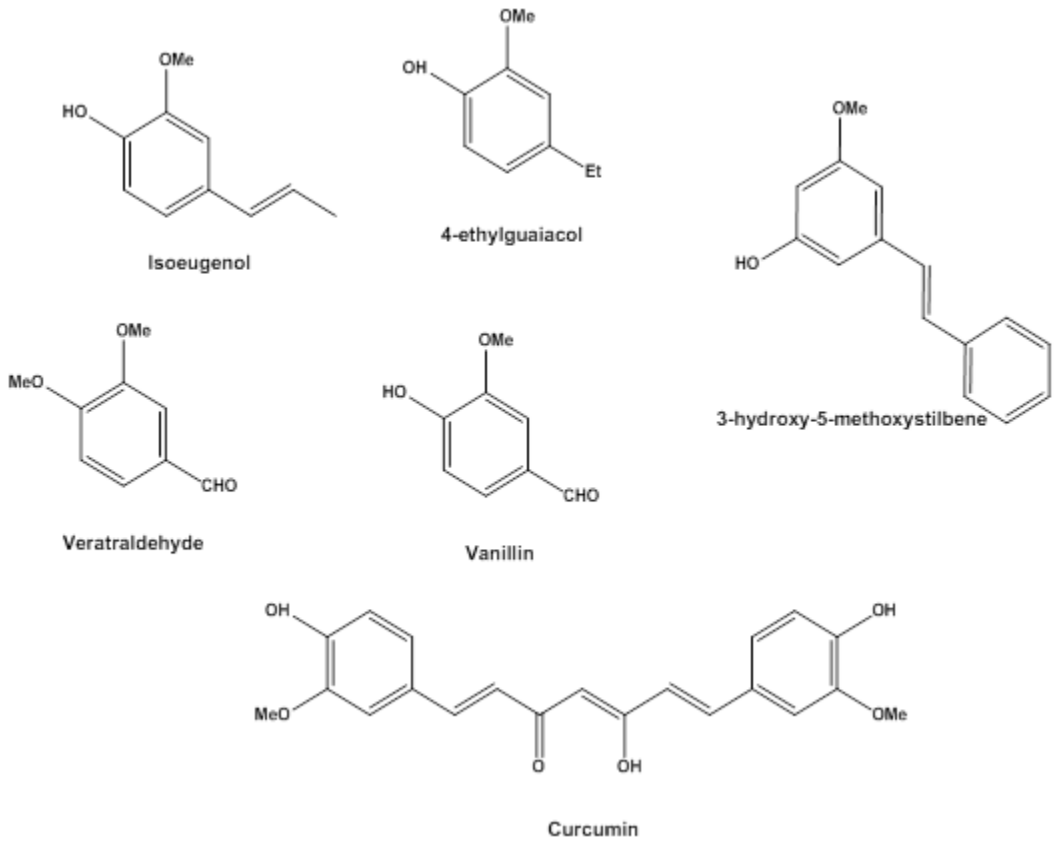


Figure 4.7 - Structure similarity of MAO inhibiting phytochemicals.

Many MAO inhibitors screened for potential neurological treatments have been isolated from natural sources, such as curcumin isolated from turmeric (Fig. 4.7). Curcumin is an *in vitro* MAO inhibitor of MAO types A and B (Kulkarni et al 2008) and also shares a similar, but dimerized, structure and substitutions to the inhibitory compounds presented here. Other MAO inhibitors include coumarin lacinartin from *Zanthoxylum schinifolium* stems (Jo et al 2002), several plant xanthones (Suzuki et al 1981) and alkaloids (Lee et al 2003, Naoi and Nagatsu 1987), and the flavonoids formononetin and kushenol isolated from the roots of *Sophora flavescens* (Hwang et al 2005). Methylaplysinopsin, an aplysinopsin isolated from the marine sponge *Aplysinopsis recticulata* is also found to selectively inhibit MAO-A in both male mice (*in vitro* and *ex vivo*) and rats (*ex vivo*) (Baird-Lambert et al 1982).The strongest of the MAO inhibitors screened in this study was isoeugenol, an odorant commonly found in spices and other foods. Isoeugenol has been shown to affect

osmoregulation and respiration in rainbow trout when used as a sedative (Olsvik et al 2007). From a natural products perspective, it has been described as a strong antioxidant (Rajakumar and Rao 1993); a potential topical acaricide to treat scabies (Pasay et al 2010); a potential anticancer compound, along with several of its derivatives (Kalmes et al 2006, Tatsuzaki et al 2006); and a potential food additive to help in the treatment of stomach ulcers (Bergonzelli et al 2003). Demetriades et al (2005) suggest isoeugenol may activate serotonin receptors due to its structural similarity with other serotonin agonists, but no research has been conducted to test this hypothesis.

While the primary goal of testing MAO activity was to identify inhibitors, I also observed stimulation of this enzyme by some of the tested phytochemicals (Fig. 4.3). This stimulation may be explained through conformational changes to the mitochondrial membranes to which MAO is bound or to conformational changes to the enzyme itself. These changes could result in the exposure of more of the enzyme's active sites with which to metabolize its substrate or to an increased affinity of substrate to the enzyme. Alternatively, these stimulatory chemicals may act by altering membrane permeability and thus substrate access to the enzyme. These potential modes of action are suggested in research by Gawienowski et al (1982) and Banerji et al (1977) where they observed increased *in vitro* MAO activity caused by tetrehydrocannabinol (THC) found in cannabis.

4.4.2 Glutamic acid decarboxylase

Balsam fir, black spruce, white spruce, tamarack, eastern hemlock, white pine, jack pine, and white cedar are all feedstocks of the Canadian pulp and paper industry. The ability of balsam fir, white spruce, tamarack, jack pine, eastern hemlock, and white cedar to inhibit GAD has reproductive implications to fish exposed to pulp mill effluents. The neurotransmitter GABA is an important stimulator of GnRH and LH release in fish (Popesku et al 2008, Trudeau 1997), therefore reductions in GAD activity could result in decreased GABA in the brain and a subsequent loss of GABA's stimulatory effect of the reproductive axis.

All of the resin acids were inhibitory to GAD, suggesting a structure-activity relationship due to their shared structural three-ringed diterpene backbone and similar substitutions (Fig. 4.8). Resin acids are ubiquitous to conifers, with specific ones being described in the wood of all the important Canadian feedstock species with the exception (to

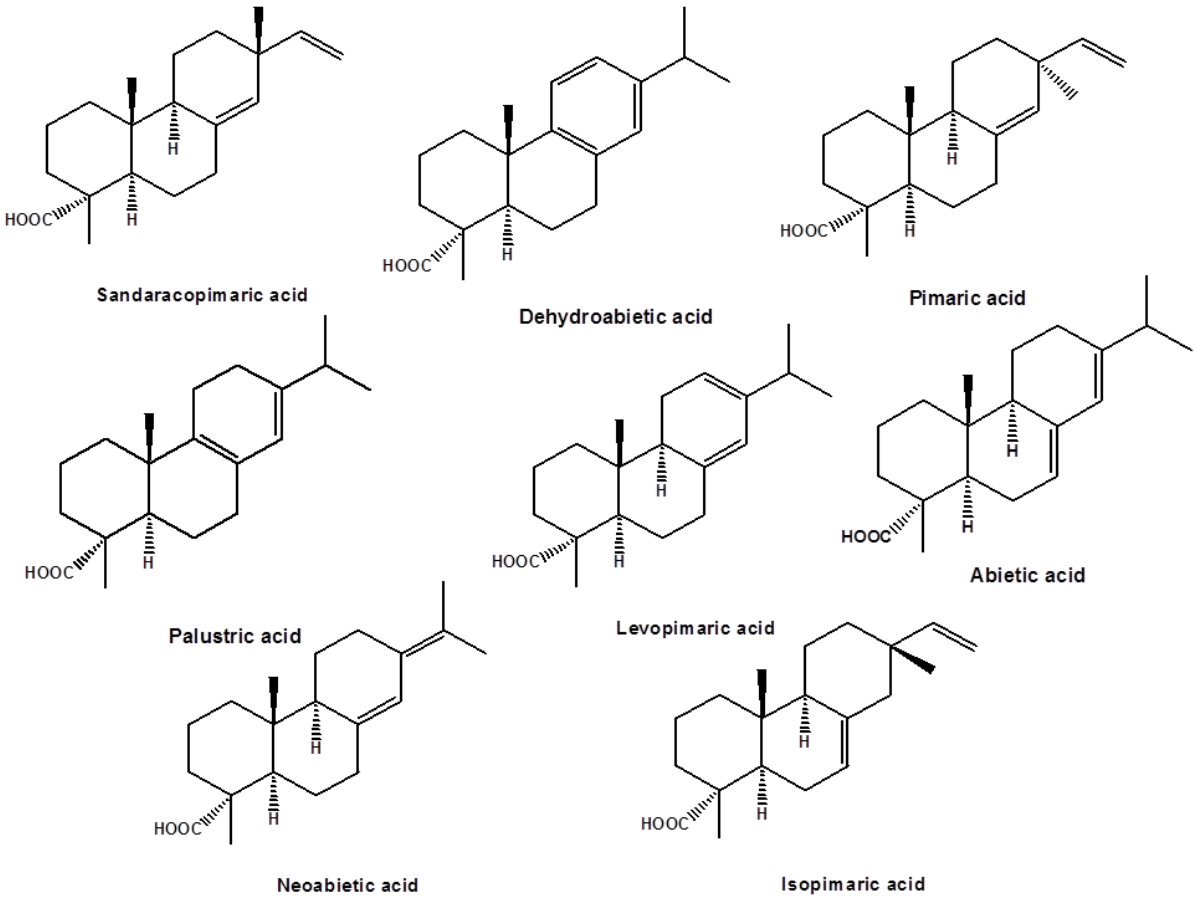


Figure 4.8 - Structure similarity of the GAD inhibiting resin acids.

my knowledge) of eastern hemlock (Table 4.2). Another of the active phytochemicals, pinosylvin, a commonly produced pre-infectious stilbene, is also present in the wood of these feedstock species (Table 4.2). Pinosylvin also has reported antifeeding, antibacterial, and antifungal activities (Clausen et al 1986, Park et al 2011). It has been described in effluents and is estrogenic in breast cancer cell lines, although does not stimulate vitellogenin production in trout liver (Mellanen et al 1996) or trout hepatocytes and was a weak inducer of CYP 1A1 activity in H4IIE cells (Parrott et al 2011).

Resin and fatty acids were a major source of toxicity for Canadian fish downstream of mills until secondary treatment was introduced systematically across the country (Ali & Sreekrishnan 2001, Bicho et al 1995, Hewitt et al, 2006; Munkittrick et al 1998). While secondary treatment systems are effective at removing resin acids, concentrations have been described in treated effluents at levels high enough to elicit chronic physiological effects in fish (Kostamo & Kukkonen 2003, Kostamo et al 2004, Leppanen et al 1998, Oikari et al

1983). For example, Oikari et al (1983) found that a 30 day exposure to 20 µg/L dehydroabietic acid led to increased spleen to body weight ratio and an increased H-type to M-type ratio of lactase dehydrogenase in 2 year-old male and female rainbow trout. Elevated concentrations of resin acids can be observed in discharged effluents after mill shut downs, after a black liquor spill within the mill, or upon release from sediments with high levels of adsorbed effluent chemicals (Kostamo & Kukkonen 2003, Makris & Banerjee 2002, Meriläinen & Oikari 2008).

Resin acids may have implications for neuroendocrine disruption of reproduction in fish. For example, dehydroabietic acid concentrations were ~4 mg/L in final effluent after a resin and fatty acid spill in a pulp and paper mill in the United States (Makris & Banerjee 2002) and resin acids have been shown to bioconcentrate in rainbow trout brains by a factor of 58.5 after 4 days (water concentration 1.4 mg/L for 2 days), resulting in a final brain concentration of 82 ppm. When tested alone, dehydroabietic acid exhibited a bioconcentration factor of 30 (1.2 mg/L exposure for 4 days) in the brain of rainbow trout (Oikari et al 1982). It is important that this family of diterpenes can bioconcentrate because abietic acid was as effective as the positive control, 3-mercaptopropionic acid (95% inhibition), at inhibiting GAD and is more lipophilic than dehydroabietic acid, and thus perhaps more bioavailable. Therefore, it is tempting to speculate that low concentrations of abietic (or other) resin acids in pulp mill effluents may affect fish reproductive endpoints via bioconcentration in the brain and inhibition of GABA synthesis.

4.4.3 GABA transaminase

None of the conifer extracts nor any of the phytochemical standards were found to change rat GABA-T enzyme activity *in vitro* compared to controls. Phytochemicals previously identified for their potential for treating anxiety, epilepsy, or other related neurological disorders via GABA-T inhibition include rosmarinic acid, found in *Melissa officinalis L.* (Awad et al 2009).

4.4.4 GABA(A)-BZD receptor binding

While the methanol extract of juniper and the ethylacetate extract of white spruce were able to bind to the benzodiazepine-binding site of the GABA(A) receptor, this *in vitro* measure of GABA(A)-BZD binding does not distinguish between agonists or antagonists and further mechanistic work is required to

determine the neuroactivities of these two species at this site. Anxiolytic GABA(A)-BZD ligands have recently been identified in traditionally used medicinal plants, such as the compounds scutellarin and betulinic acid which are found in *Scutellaria lateriflora* (Awad et al 2003) and *Souroubea sympetala* (Mullally et al 2011), respectively.

4.4.5 Boreal conifer neuroendocrine disruptors as natural medicinal products

I performed these assays using two separate model species (goldfish and rat). Given the conservation of function of the GABAergic and dopaminergic systems between fish and mammals (Popesku et al 2008) I expect that the results from one vertebrate lineage to have implications on the other (i.e., that compounds that affect neurological endpoints in fish would act similarly in mammals, and vice-versa). For future research, I would suggest testing MAO and GAD activities in rat, and GABA(A)-BZD binding and GABA-T activity in goldfish in order to do a direct comparison of the bioactivities of my samples between these two vertebrate species. Furthermore, assessing other enzymes or receptors in the GABAergic or dopaminergic systems (e.g., dopamine receptors, other GABA receptors, or enzymes responsible for dopamine synthesis such as DOPA decarboxylase) could also identify other modes in which these two systems could be disrupted, although I did attempt but was unsuccessful at optimizing and validating a dopamine D2 receptor-binding assay in goldfish.

Canada is the second largest exporter of forest products in the world (after the United States) and the forest products industry remains among the top five contributors to Canadian net trade (NRCan 2013). The last five years has seen a decline in Canadian pulp and paper competitiveness and the industry is currently in a transitional phase, turning its attention to new market opportunities, value-added products, and biorefineries (Kovacs et al 2010, NRCan 2013). During the pulping process, woods are extracted of their secondary metabolites and lignins and only the cellulose is retained for the final product. Given this, conifer forests remain a potential resource of natural products as bioactive secondary plant metabolites are being discharged as waste. My data support the promise of conifer-derived compounds specifically for their use in the treatment of dopaminergic and

GABAergic related diseases or disorders. Phytochemicals from plants have the potential to affect neurological behavioural processes related to anxiety (Awad et al 2007), epilepsy (Awad et al 2009), depression (Tabassum et al 2010), and alertness (Scholey et al 2010). While the isolation of the anti-cancer pharmaceutical taxol from Pacific yew (*Taxus brevifolia*) is widely recognized as the most successful example of a phytochemical product derived from a conifer, other conifer-derived products are also be found on the market, such as the anti-inflammatory and anti-oxidant patented product Pycnogenol (www.pycnogenol.com) derived from maritime pine (*Pinus pinaster*) or several formulations of resveratrol supplements containing extracts of 'pine bark'. Conifers do not possess the level of biodiversity or complex phytochemistry seen in tropical plants, but Canadian boreal conifer forests still represent a potentially underexploited source of naturally-derived compounds with similar activities as those more often studied in tropical species.

4.5 Conclusion

This is one of the first studies to examine the potential neuroendocrine effects of conifer extracts and specific conifer-derived phytochemicals associated with pulp mill effluents. The identification of strong MAO and GAD inhibitors during the screening of these extracts and phytochemicals in this study provides an avenue for future testing and possible development of novel enzyme inhibitors. Chapter 5, and the studies I performed with Basu et al (2009) and Milestone et al (2012) demonstrate that effluents from mills using conifer feedstocks tested in this study contain compounds that are capable of affecting enzymes important for GABA synthesis and dopamine metabolism. Similar bioactivities were seen in the diversity of feedstock species tested in this study, Chapter 3, and in my collaboration with Milestone et al. (2012), indicating that bioactive chemicals may survive effluent treatment strategies.

The spruce species and balsam fir tested in this study seem to be consistently neuroactive lineages in my assays. Therefore, if reproductive effects observed *in vivo* (Kovacs et al 2007, McMaster et al 2006, Van Der Kraak et al 1992), are indeed caused by MAO or GAD inhibition, I might expect reduced

reproductive inhibition with an increased proportion of hemlock or white pine used as feedstocks at a mill, since these are species with lower MAO and GAD inhibition. White and black spruce, as well as balsam fir, represent major Canadian pulp and paper feedstocks harvested from boreal forests, while white pine and hemlock are less available and derived from mixed deciduous forest areas. I also identified resin acids as an important phytochemical group of GAD inhibitors. These are ubiquitous phytochemicals in most important Canadian conifer feedstocks and can be released to the environment when there are in-mill spills or sub-optimal effluent treatment.

I propose that further research and characterization of wood extracts (Chapter 6) could simplify and enhance the ability to identify the chemicals responsible for these effects in the chemically complex and difficult to work with mill effluents (Chapter 5). The eventual selective removal of these phytochemicals in a biorefinery context could not only provide novel uses for current waste products as potential treatments for neurological disorders, but could also benefit the environment as fish and other species downstream of pulp mills would no longer be exposed to these potential neuroendocrine disruptors.

Table 4.2. List of the tested phytochemicals and their described presence in the conifers tested in this study.

Compound	Species	Tissues
4-ethylguaiacol	White pine	Wood[1,2,3,6] resin[4,6] needles[5,6] bark[2] cones[6]
	Juniper	Unknown (tincture)[7]
	White spruce	Cones[8]
Vanillin	White pine	Wood[1,6] needles[6] resin[6] cones[6] cambial tissues[9]
	Juniper	Unknown (tincture)[7]
	White spruce	Wood[10]
	Jack pine	Wood[11,12,13,14]
	Black spruce	Wood[16] bark[15]
	Tamarack	Needles[17]
Dehydroabietic acid	Balsam fir	Wood[18] needles[19] bark[19]
	White pine	Wood[2,20] resin[6] needles[5,6] bark[2] cones[6]
	White spruce	Wood[23] resin[24,25] needles[23] bark[23] cones[22,23]
	Jack pine	Wood[14,23,33] needles[23,30,31] bark[23] cones[23]
	Black spruce	Wood[3,18] bark[15]
	White cedar	Wood[34] bark[34]
	Tamarack	Wood[35,36] needles[37]

Table 4.2 (continued). List of the tested phytochemicals and their described presence in the conifers tested in this study.

Compound	Species	Tissue
3-hydroxy-5-methoxystilbene	Balsam fir	Wood[28]
	White pine	Wood[20,39,40,41,42,43]
	White spruce	Wood[44] bark[43]
	Jack pine	Wood[11,14,33,40,44,45,46,47] bark[44]
	Black spruce	Wood[44] bark[44]
	White cedar	Wood[28,44,45,48] bark[44]
	Tamarack	Wood[44] bark[44]
	Eastern hemlock	Wood[44] bark[44]
Abietic acid	Balsam fir	Wood[18] needles[19] bark[19]
	White spruce	Wood[50,51] resin[24,25] needles[51] bark[50,51] cones[22,23]
	Jack pine	Wood[3,14,33] needles[30,31] cones[23]
	Black spruce	Wood[3,18] bark[15]
	White cedar	Wood[34] bark[34]
	Tamarack	Wood[35,36] needles[37,52]
Isopimaric acid	Balsam fir	Wood[18] needles[19] bark[19]
	White pine	Wood[2,20] resin[6] needles[5,6] bark[2] cones[6]
	Juniper	Needles[53,56] berries[54,55,57]
	White spruce	Resin[24,25] cones[23]
	Jack pine	Wood[14,23,33] needles[23,30] bark[23] cones[23]
	Black spruce	Wood[3,18] bark[15]
	White cedar	Needles[58] twigs[58]
	Tamarack	Wood[35,36] needles[37]

Table 4.2 (continued). List of the tested phytochemicals and their described presence in the conifers tested in this study.

Compound	Species	Tissue
Levopimaric acid	Balsam fir	Wood[18] needles[19] bark[19]
	White pine	Wood[2] needles[5] bark[2]
	White spruce	Resin[24] cones[22]
	Jack pine	Wood[33] needles[30]
	Black spruce	Wood[18]
	Tamarack	Needles[52]
Manool	Balsam fir	Wood[27]
	White pine	Needles[59]
	Juniper	Wood[63] needles[63] berries[60,62,63] root[63]
	White spruce	Resin[25]
	Tamarack	Twigs[64]
Neoabietic acid	Balsam fir	Wood[18] needles[19] bark[19]
	White pine	Wood[2,3] needles[5] bark[2] cone[61]
	White spruce	Resin[24,25] cones[22]
	Jack pine	Wood[14,33] needles[30, 31]
	Black spruce	Wood[18]
	White cedar	Wood[34] bark[34]
	Tamarack	Wood[35,36]
Palustric acid	Balsam fir	Wood[18] needles[19] bark[19]
	White pine	Wood[2] resin[6] needles[5,6] bark[2] cones[6]
	White spruce	Resin[24,25] cones[22]
	Jack pine	Wood[33] needles[30]
	Black spruce	Wood[3,18] bark[15]

Table 4.2 (continued). List of the tested phytochemicals and their described presence in the conifers tested in this study.

Compound	Species	Tissue
	Balsam fir	Wood[18,29] needles[19] bark[19]
	White pine	Wood[2,3] resin[6] needles[6] bark[2] cones[6]
Pimaric acid	White spruce	Needles[49]
	Jack pine	Wood[3,14,33]
	Black spruce	Wood[18] bark[15]
	White cedar	Needles[38] twigs[38]
	Balsam fir	Wood[28]
	White pine	Wood[40,41,42] bark[32]
	White spruce	Wood[44] bark[44]
Pinosylvin	Jack pine	Wood[11,14,33, 40,44,45,46,47] bark[44]
	Black spruce	Wood[44] bark[44]
	White cedar	Wood[28,44,45,48] bark[44]
	Tamarack	Wood[44] bark[44]
	Eastern hemlock	Wood[44] bark[44]
	Balsam fir	Wood[18] needles[19] bark[19]
	White pine	Wood[2] resin[6] needles[5,6] bark[2,6]
	Juniper	Needles[56,57] berries[54,55,57]
Sandaracopimaric acid	White spruce	Resin[24,25]
	Jack pine	Wood[14] needles[30]
	Black spruce	Wood[18] bark[15]
	Tamarack	Wood[36] needles[37]

Table 4.2 (continued). List of the tested phytochemicals and their described presence in the conifers tested in this study.

Compound	Species	Tissue
Veratraldehyde	Balsam fir	Branch tips[26]
	Jack pine	Branch tips[26]
	Black spruce	Branch tips[26]
Isoeugenol	Juniper	Berries[21]
	Black spruce	Bark[15]

[1]Cheng et al 2010; [2]Santamour 1967; [3]Conner et al 1980; [4]Duke 1992; [5]Zinkel & Magee 1987; [6]Oros & Simoneit 2001; [7]Iordache et al 2009; [8]Pepper & Supathna 1978; [9]Higuchi et al 1960; [10]Perng 1986; [11]Poncsak et al 2009; [12]Heil & Lindsay 1990; [13]Polcin & Rapson 1971; [14]Von Rudloff & Sato 1963; [15]Pakdel et al 1994; [16]Jayne 1953; [17]Niemann 1972; [18]O'Conner et al 1992; [19]Degtyarenko et al 1998; [20]Geraldo de Carvalho et al 1996; [21]Loziene et al 2010; [22]Kersten et al 2006; [23]Eberhardt et al 1994; [24]Abbott et al 2010; [25]Tomlin et al 2000; [26]Nierop et al 2005; [27]Manville 1975; [28]Willfoer et al 2003; [29]Demers et al 2000; [30]Schuh & Benjamin 1984; [31]Ikeda et al 1977; [32]Raiber et al 1995; [33]Sinclair & Dymond 1973; [34]Gilbert et al 1970; [35]Powell & Raffa 1999; [36]Wagner et al 1983; [37]Ohigashi et al 1981; [38]Sulea & Leca 2009; [39]Lindstedt 1949; [40]Lindstedt & Misiorny 1951; [41]Erdtman 1944; [42]Erdtman 1943; [43]Suga et al 1993; [44]Pietarinen et al 2006; [45]Lindberg et al 2004; [46]Campbell & Ellis 1992; [47]Phelan et al 2009; [48]Kokubo et al 1990; [49]Bedon et al 2010; [50]LaFlamme 1979; [51]Meyers et al 1995; [52]Michl et al 1988; [53]De Pascual et al 1980; [54]De Marino et al 2011; [55]Martin et al 2006; [56]San Feliciano et al 1991; [57]De Pascual et al 1973; [58]Chang et al 2000; [59]Krauze-Baranowska et al 2002; [60]Vichi et al 2008; [61]Deyoe & Brown 1979; [62]Vichi et al 2007; [63]Gonny et al 2006; [64]Von Rudloff 1987

Chapter 5: *In vitro* GAD inhibition and chemical characterization of a reproductively inhibitory thermomechanical pulp mill effluent

[1]Andrew Waye, [1]Ammar Saleem, [1]Jose-Antonio Guerrero-Analco, [1]Jingqin Mao, [1]John T Arnason, [1]Vance L Trudeau

[1]Department of Biology, University of Ottawa, Ottawa, ON, K1N 6N5

This study examined novel methods by which to extract and chemically analyze pulp mill effluents with the goal of identifying active putatives responsible for similar GAD inhibition as a potential mode by which neuroendocrine disruption of reproduction in fish may occur. Bioassay-guided fractionation, targeted chemical analysis, and discriminant metabolomic analysis were employed to characterize the neuroactivity of neuroactive effluent fractions.

Statement of author contributions

AW, JTA, and VLT conceived and designed this study with contributions from JAGA and AS. Samples were prepared by AW, JAGA, and JM and tested in bioassays by AW. Chemical analyses were performed by AW and AS. AW performed analysis of data and prepared manuscript with contributions from JTA and VLT.

5.1 Introduction

Pulp mill effluent extracts from a thermomechanical pulp (TMP) mill in eastern Canada were able to interact with key neurotransmitter systems important in the control of reproduction in fish (Basu et al 2009). Neuroactivities associated with mill effluents have also been demonstrated from extracts of other mills of varying types in Canada, New Zealand, and Brazil (Milestone et al 2012), as well as extracts of both hardwood and softwood pulping feedstocks (Basu et al 2012, Milestone et al 2012, Waye et al 2014a; Chapters 3 and 4). This research indicates that the disruption of neuroendocrine function could play an important role in lab and field studies where reproductive impairment is observed in fish that are exposed to pulp mill effluents by mills that use various pulping methods, effluent treatment systems, and feedstock species (Waye & Trudeau 2011).

In this chapter, I attempt to identify active principles in TMP effluent extracts that may be responsible for the inhibition of the enzyme glutamic acid decarboxylase (GAD) using bioassay-guided fractionation. The enzyme GAD is responsible for synthesizing γ-aminobutyric acid (GABA) from glutamic acid. GABA in turn is an important neuroendocrine stimulator of the reproductive axis in fish (Popesku et al 2008, Trudeau 1997). I have previously identified the diterpenoid resin acids as a particularly interesting group of phytochemicals that inhibit GAD (Waye et al 2014a; Chapter 4) since they can be discharged in effluent where spills are present or secondary treatment has been disrupted (Kostamo & Kukkonen 2003, Makris & Banerjee 2002, Meriläinen & Oikari 2008). They have the ability to bioconcentrate in the brains of fish after waterborne exposure (Oikari et al 1982), and have other described neuroactivities (Nicholson 1994, Zheng & Nicholson 1996, Zheng & Nicholson 1998).

Isolating and identifying biologically active chemicals in pulp mill effluents is challenging, due to the high chemical complexity and low yields of individual substances in extracts of pulp effluents (Hewitt et al 2006). To accomplish our goal of successfully identifying GAD inhibitors in chemically complex and dilute samples, I used two novel techniques in effluent preparation and extraction: spray drying and reverse osmosis. As pulp mill effluents are highly dilute aqueous solutions, extraction of compounds or removal of large volumes of water from the effluent for chemical testing and analysis is both time and energy consuming. Spray drying is a mode by which an aqueous sample can be quickly

brought to dryness by nebulizing the sample at high pressure through a fine nozzle where the droplets are evaporated upon contact with a stream of hot air. This results in a highly concentrated effluent solid with which conventional extraction techniques can then be performed. Reverse osmosis allows for the fast concentration of large volumes of effluent by using pressure and a membrane through which only water (and smaller) molecules may pass. Once the effluent has been concentrated several fold, chemical recovery from the retentate is more easily performed. Both of these methods circumvent the need to use the large amounts of solvents that would be necessary for liquid-liquid extraction of unconcentrated effluents and avoid the limitations of performing solid phase extractions, where the stationary adsorbent phase can quickly become saturated by the complex effluent mixture.

I used three approaches to identify bioactive compounds from effluent extracts, the first of which was bioassay-guided fractionation. Bioassay-guided fractionation is performed by the identification and successive fractionation of bioactive samples to the point where single bioactive molecules are isolated and identified. The second approach was targeted chemical analysis, where previously identified bioactive compounds can be screened for in bioactive samples. The third, and novel, approach for the analysis of pulp mill effluents was discriminant analysis using metabolomics with ultra-high-performance liquid chromatography, time-of-flight mass-spectrometry (UPLC-QTOF), which compares active and non-active samples and identifies metabolites unique to the active samples through computation. These metabolites can then be characterized and tested for bioactivity. In this study, I use the above approaches with the goal of successfully identifying GAD inhibitors.

5.2 Methods

5.2.1 Glutamic acid decarboxylase (GAD) activity assay

The GAD assay was performed using previously published methods (Awad et al 2009). Briefly, male and female goldfish (*Carassius auratus*; Aleong's International Inc., Mississauga, ON, Canada) were anesthetized with MS-222 (Syndel Laboratories, Qualicum, BC, Canada), decapitated, and whole brains were dissected, combined, and stored at -80 °C. Whole goldfish brains were thawed and then homogenized in 1:10 (w/v) 10 mM phosphate buffer solution (pH 7.5) and stored in 1 ml aliquots at -80°C. The concentration of protein in all samples was determined using the method of Bradford (1976).

To measure GAD enzyme activity, 50 μg of brain homogenate was re-suspended in glass tubes to a final volume of 350 μl of 10 mM phosphate buffer (including 60 μM pyridoxal-5'-phosphate and 120 μM dithiothreitol, pH 7.4). The reaction was initiated by the addition of 30 mM glutamate containing 0.1 μCi L-[1-^{14}C] glutamic acid (50.0 mCi/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO, USA) and a suspended Whatman GF/B filter paper soaked in Scintigest (Fisher Scientific, Ottawa, ON, Canada). Following a 45 min reaction at 37°C, the reaction was terminated by the addition of 0.5 ml of 0.25 M HCl. The vials were allowed to incubate for another 60 min and the radioactivity (released CO_2) trapped on the filter paper was determined. All extracts were tested in triplicate at a final concentration of 50 μg/ml in DMSO and the GAD inhibitor 3-mercaptopropionic acid (1 mM) was used as a positive control.

Statistical differences ($p < 0.05$) in activities were detected using 1-way ANOVA, normality, and Levene's test in SPSS (IBM, Armonk, NY, USA) with a Tukey or Dunnett's T3 (when homogeneity of variance was not observed) post-hoc test.

5.2.2 Pulp mill effluents

Mill primary and secondary effluents were sampled from a representative thermomechanical pulp (TMP) mill in Eastern Ontario (Tables 5.1 and 5.2). The effluent was first subjected to primary treatment in a clarifier and then was subjected to secondary treatment in a conventional activated sludge treatment plant. The primary feedstocks were balsam fir (*Abies balsamea* L.; 75%) and black and white spruce (*Picea mariana* (Mill.) BSP and *P. glauca* (Moench); 25%), and effluents from this mill are known to be inhibitory to reproduction in the 5-day fathead minnow egg production assay at FPInnovations labs in Pointe-Claire, QC, Canada (Popesku et al 2010).

5.2.2.1 Primary effluent

One 20 L bucket of effluent was grab sampled from the primary clarifier outflow of the TMP mill and shipped to the University of Ottawa and kept at 4^{o}C for two months until spray dried. One litre of primary effluent was spray dried with a Buchi Mini Spray Drier B-191 (Buchi Labortechnik AG, Flawil, Switzerland) at a rate of 40 ml/h (inlet 100^{o}C, outlet 42^{o}C, aspirator 65%, pump 20%, airflow 600 L/h). The spray-dried material was reconstituted in 200 ml of water and liquid:liquid solvent series extraction was performed

Table 5.1. Pulp mill description and operating procedures.

Pulping	Brightening agent	Furnish	Product	Production	Water usage	Primary treatment	Secondary treatment
TMP deinked pulp	$Na_2S_2O_4$ / Na_2SO_3	75% fir, 25% spruce	Newsprint	750 t/d	19 m^3/t	Primary clarifier	24h activated sludge

TMP, thermomechanical pulp

Table 5.2. Measured physicochemical parameters of tested effluents.

Date sampled	pH	Conductivity	Hardness	DO	NH_3	BOD	COD	SS	RFA
Oct 1, 2009	6.2	868 µS	135 mg/L	7.4 mg/L	3.44 mg/L	23 mg/L	404 mg/L	43.6 mg/L	0.01 mg/L

DO, dissolved oxygen; BOD, biological oxygen demand; COD, chemical oxygen demand; SS, suspended solids; RFA, resin and fatty acids (detection limit 0.01 mg/L).

using gentle rocking in a 1 L separatory funnel with first hexane (x 2 and then combined) then ethylacetate (x 2 and then combined). Solvent extracts were brought to dryness by rotoevapotation (40^{o}C) while the aqueous phase was dried by lyophilisation. A 5 mg aliquot of the original spray dried effluent was saved for testing GAD activity.

For fractionation of the primary effluent ethylacetate extract, 1.3 g of extract was adsorbed to 30 g of silica and run through a glass column containing 300 ml of silica gel. Eluates were collected in 100 ml volumes, brought to dryness in a rotary evaporator, and analyzed by thin layer chromatography (TLC) on silica gel eluted by an appropriate mixture of hexane-ethylacetate, ethylacetate, or ethylacetate-methanol to allow proper chromatographic separation. TLC bands were visualized using a UV lamp (short λ = 254 nm, long λ = 325 nm) and developed with ceric sulfate solution (5% of H_2SO_4) for oxidization. Eluates with similar TLC banding patterns were combined to yield a total of 11 chemically distinct pooled fractions which were then brought to dryness by rotoevaporation.

5.2.2.2 Secondary effluent

A 1000 L grab sample was taken from the outlet pipe of the TMP mill and shipped to FPInnovations labs (Pointe-Claire, QC) where they were stored at 4^{o}C for one week until concentrated. A 90 L aliquot of secondary effluent was concentrated to 2.5 L by reverse-osmosis using an AFC99 polyamide film membrane (Xylem Inc., Rye Brook, NY) in 5 hours with a system pressure of 20 bar. A 1:1 volume solvent series liquid:liquid extraction was performed on the 2.5 L of concentrate, with two hexane extractions and three ethylacetate extractions performed. The extracts were rotoevaporated at 40^{o}C to dryness and the aqueous phase was dried by lyophilisation. Extractions with the same solvents were not combined before testing in assay as they appeared visually different.

5.2.3 Chemical analysis of samples

For UPLC-QTOF analysis, separation of analytes was achieved using an Acquity BEH C18 1.7um 2.1 x 100mm column (Waters Corporation, Milford, MA) connected with a VanGuard Pre-column 2.1 x 5mm (Waters Corporation, Milford, MA). Column temperature was set to 50°C and sample temperature was maintained at 10°C. The mobile phases were, A, water + 0.1% formic acid, and B, acetonitrile + 0.1% formic acid (Fisher Optima LC-MS, Fisher Scientific Company, Ottawa, ON), with a flow rate of 0.5 ml/min. The mobile phase

composition was 0-1 min 5% A isocratic, 1-6 min linear gradient 5-50% B, 6-8 min 50-95% B, 8.01-10 min 5% A isocratic (total run time 10 min). Sample injection conditions: 1 uL injection followed by a strong wash 200 uL (90% acetonitrile + 10% water) and weak wash 600 uL (10% acetonitrile + 90% water). QTOF analysis was carried out on a Waters Xevo G2 instrument under the following conditions: MassLynx software, MSe ESI+ mode, lock mass Leucine Enkephalin 12C 556.2615, source temperature 120°C, desolvation temperature 400°C, cone gas (N_2) flow 50 L/hr, desolvation gas (N_2) flow 1195 L/hr. MSe conditions, mass range 100-1500 Da, F1 CE, 6V, F2 CER 10-30 V, cone voltage 20 V, scan time 1 sec., calibration 50-1000 Da sodium formate. Principal component analysis (PCA) and discriminate analysis (OPLS-DA) were performed by MassLynx (version 8.03). Compound identification was undertaken by comparing elemental composition and isotopic fit of target compounds using MassLynx software and finding matches in the METLIN Metabolomics Database (Scripps Research Institute, San Diego, USA) and ChemSpider (Royal Society of Chemistry, London, UK). Mass fragmentation was carried out using high energy spectrum to confirm the identification using MassFragment software.

Table 5.3. Comparison of primary effluent extract yields of different extraction methods

	Fraction	Extract yield (mg/L)
Spray dry + solvent series	Hexane	90
	Ethylacetate	1300
	Aqueous phase (freeze dried)	1100
Solvent series[1]	Hexane	12
	Ethylacetate	200
	Aqueous phase (freeze dried)	2600
Polyphenolic[1]	Ethanol	150
	Aqueous phase (freeze dried)	2180

[1] Yields reported in Basu et al (2009)

Table 5.4. Comparison of secondary effluent extract yields of different extraction methods

	Fraction	Extract yield (mg/L)
Reverse osmosis + solvent series	Combined hexane	0.8
	Combined ethylacetate	5.5
	Aqueous phase (freeze dried)	39.7
Solvent series[1]	Hexane	10
	Ethylacetate	30
	Aqueous phase (freeze dried)	2720
Polyphenolic[1]	Ethanol	90
	Aqueous phase (freeze dried)	2000

[1]Yields reported in Basu et al (2009)

5.3 Results

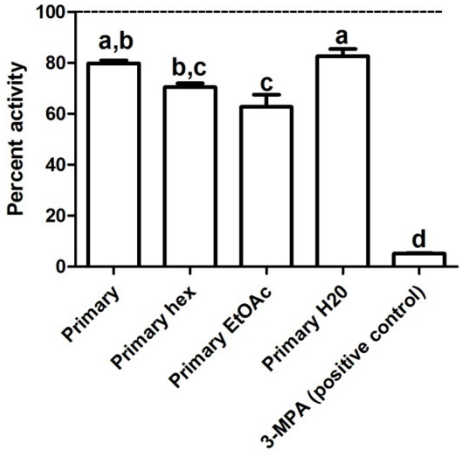


Figure 5.1 - Effects of primary effluent fractions on in vitro GAD activity expressed as mean percent activity of controls. Error bars represent one standard error. Extracts tested in triplicate with final concentration of 50 μg/ml. Letters denote statistical differences ($p < 0.05$). Dotted line denotes solvent control activity (100%). Hex, hexane; EtOAc, ethylacetate; H2O, effluent aqueous phase; 3-MPA, 3-mercaptopropionic acid.

5.3.1 Effluent extract efficiencies

The extraction efficiencies varied greatly by method. Spray-drying primary effluent before extraction resulted in yields of 90 mg/L for hexane extraction and 1300 mg/L for ethylacetate extraction. The freeze dried aqueous phase yield was 1100 mg/L (Table 5.3). The reverse osmosis (RO) concentration of 90 L of secondary effluent before solvent series extraction resulted in much lower yields on a per litre basis with 1 mg/L for hexane extraction and 6 mg/L for ethylacetate extraction. The freeze dried aqueous phase yield from concentrated secondary effluent was 39 mg/L (Table 5.4).

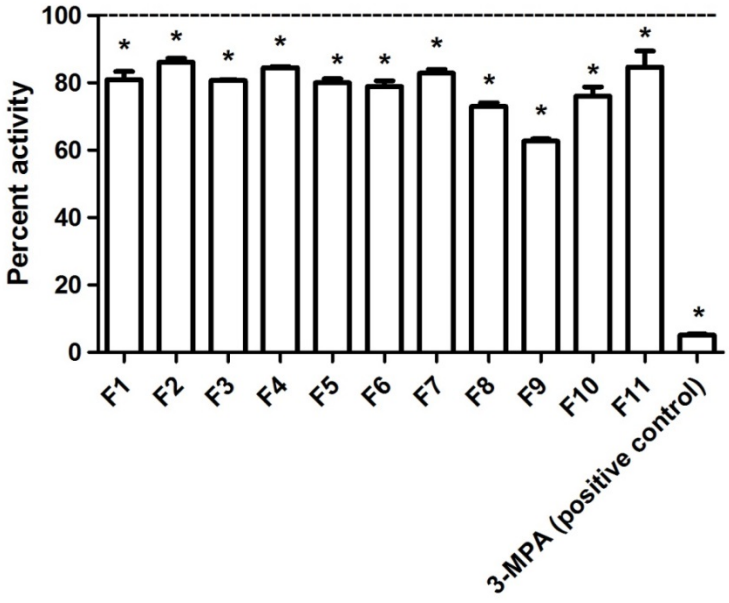


Figure 5.2 - Effects sub-fractions of ethylacetate fractions of primary effluent on in vitro GAD activity expressed as mean percent activity of controls. Error bars represent one standard error. Extracts tested in triplicate with final concentration of 50 μg/ml. Letters denote statistical differences ($p < 0.05$). Dotted line denotes solvent control activity (100%).

5.3.2 Glutamic acid decarboxylase inhibition by primary effluent extracts

Reconstituted spray dried effluent was inhibitory to GAD activity compared to controls (20.3% inhibition; Fig. 5.1), and was not significantly different than the activity of the aqueous phase (17.4% inhibition) of the liquid-liquid extracts. From the liquid-liquid extraction, the hexane (29.6% inhibition) and ethylacetate (37.3% inhibition) fractions had the highest GAD inhibition (Fig. 5.1). The ethylacetate extract also had sufficient yield with which to perform fractionations and all of the resulting fractions (F1 – F11) significantly inhibited GAD compared to vehicle controls (14.0 – 37.3% inhibition; Fig. 5.2), however F9 contained the highest bioactivity (37.3% inhibition). All of these fractions showed considerable chemical complexity upon HPLC analysis, absence of resin acids, and low yields. Since all pulp effluents now undergo secondary treatment, it was decided to concentrate efforts on these.

5.3.3 Glutamic acid decarboxylase inhibition by secondary effluent extracts

Only two extracts of RO concentrated secondary effluent were active: these were the first and second ethylacetate liquid-liquid extracts (48.0% and 43.5% inhibition,

respectively; Fig. 5.3). Neither the third ethylacetate liquid-liquid extract, nor the hexane extracts or aqueous phase differed from vehicle controls.

5.3.4 Secondary effluent discriminant chemical analyses

A discriminant analysis was performed comparing the metabolic profiles of the two active ethylacetate extracts to those of the inactive extracts (Fig. 5.4) and in the resulting S-Plot three "marker" metabolites unique to the active fractions were identified. One of these three distinctive chemical markers was found using negative ionization of the sample (Fig. 5.4a), while the other two markers were found with positive ionization (Fig. 5.4b). Several putative compound matches for each accurate mass were found for each marker using MassLynx (Table 5.5). Several compounds in the matches were obtained commercially, but only 4-acetylpyridine (Fig. 5.5) was confirmed by matching retention time, exact mass, and fragmentation pattern, using UPLC-QTOF. However, when tested in the GAD assay, 4-

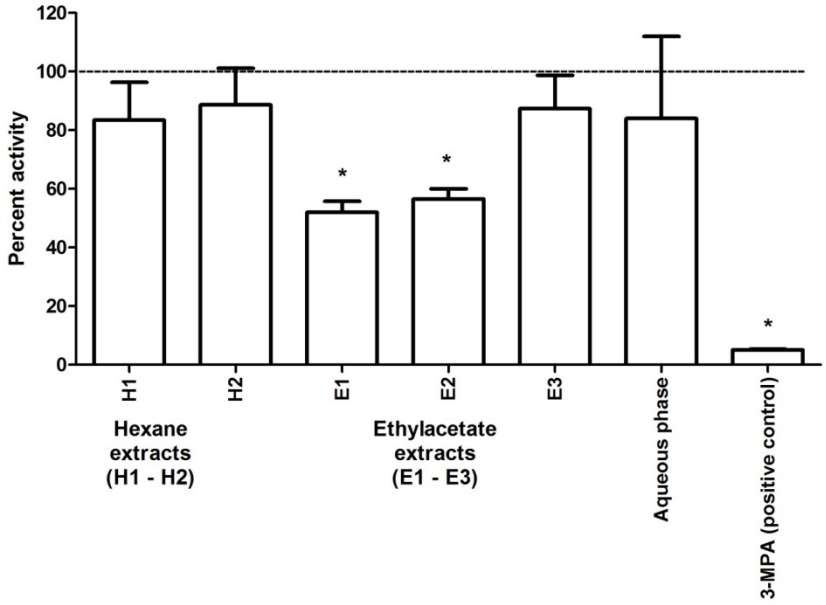


Figure 5.3 - Effects secondary effluent fractions on *in vitro* GAD activity expressed as mean percent activity of controls. Error bars represent one standard error. Extracts tested in triplicate with final concentration of 50 µg/ml. Asterisk denote statistical differences from solvent controls ($p < 0.05$). Dotted line denotes solvent control activity (100%).

acetylpyridine (1 mM) had no effect on GAD activity (data not shown) when compared to vehicle controls.

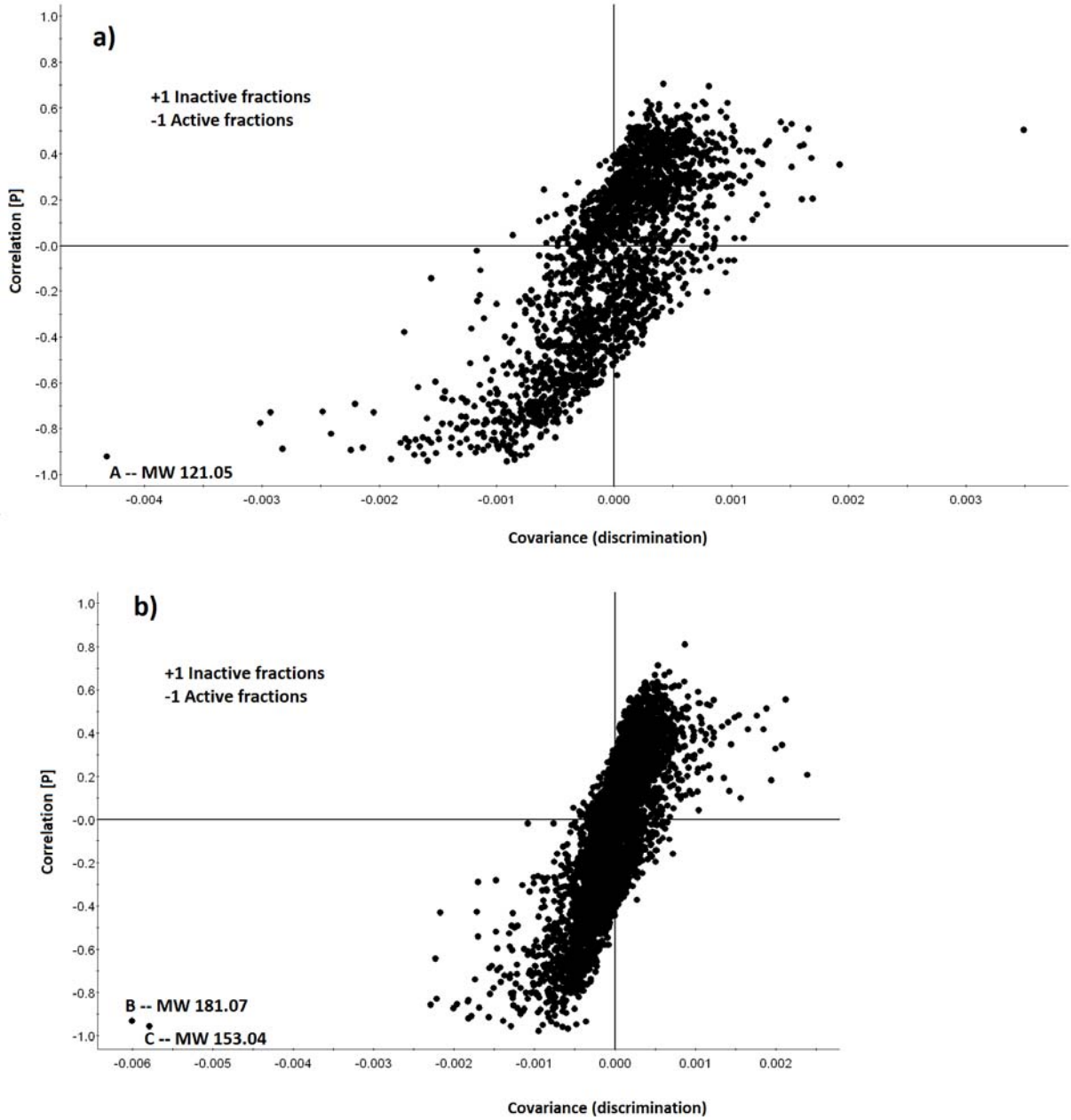


Figure 5.4 - Identification of three metabolites unique to GAD inhibitory secondary effluent fractions as determined by discriminant metabolomic analysis. Metabolites (denoted by dots) in the lower left quadrants are those most unique to active ethylacetate fractions E1 and E2. a) Discriminant analysis performed on metabolites ionized in negative ionization mode. b) Discriminant analysis performed on metabolites ionized in positive ionization mode. The mass to charge ratio of metabolites (A, B, and C) unique to active fractions are indicated next to their identification.

I also performed targeted analysis of secondary effluent samples for dehydroabietic acid, abietic acid, veratraldehyde, vanillin, 3-hydroxy-5-methoxystilbene, and pinosylvin. These phytochemicals have been found in effluent and/or their various waste streams and I

previously found neuroactivities with them including GAD and MAO inhibition (Waye et al 2014a; Chapter 4). Hexane extract 1 contained both dehydroabietic and abietic acid, while hexane extract 2 only contained dehydroabietic acid. Vanillin was the only one of these phytochemicals to be found in the ethylacetate extracts 1 – 3. These phytochemicals identified by targeted chemical analysis were not quantified due to their low concentration and co-elution with other compounds.

Table 5.5. List of putative molecular formulae and metabolite identification for three chemical markers identified by MassLinx as discriminant to GAD-inhibitory fractions. Compounds in bold were those available for purchase and screened for confirmation using co-chromatography, accurate mass, fragmentation pattern, and isotopic fit. Metabolites A, B, and C are those three identified in Figure 5.4 as unique to active fractions.

Ionization mode	Metabolite mass and putative molecular formula	Tentative identification	Identification confirmed?
Negative	A Observed mass: 121.05 g/mol C_7H_7NO	**Benzamide**	No
		2-Amino-2-(hydroxymethyl)-1,3-propanediol	No
		2,6-Dimethylaniline	No
		N-Methyl-1-phenylmethanamine	No
		N-Ethylaniline	No
		2,3,5-Trimethylpyridine	--
		4-acetylpyridine	Yes
		2-acetylpyridine	--
		2-Ethyl-6-methylpyridine	--
Positive	B Observed mass: 181.07 g/mol $C_9H_{11}NO_3$	**3-Hydroxyphenylalanine**	No
		D-Tyrosine	No
		3,5-Dimethoxybenzamide	--
		2,6-Dimethoxybenzamide	--
		Methyl 4-amino-2-methoxybenzoate	No
		2-Phenylserine	--
		2-Isopropyl-6-nitrophenol	--
		1-Methoxy-2,3-dimethyl-4-nitrobenzene	--
		Methyl 4-amino-3-methoxybenzoate	--
		2-(4-Methoxyphenoxy)acetamide	--
	C Observed mass: 153.04 g/mol $C_7H_7NO_3$	**5-Amino-2-hydroxybenzoic acid**	No
		4-Amino-2-hydroxybenzoic acid	No
		2-Methyl-4-nitrophenol	No
		4-Methyl-2-nitrophenol	No
		(2-Nitrophenyl)methanol	--
		5-Methyl-2-nitrophenol	--
		4-Methyl-3-nitrophenol	--
		3-Methyl-4-nitrophenol	--
		3-Methyl-2-nitrophenol	--
		3-Amino-4-hydroxybenzoic acid	No

Tentative identifications listed in order presented by MassLynx analysis, with descending confidence. Compounds that could not readily be obtained commercially are denoted by hashes (--).

5.4 Discussion

5.4.1 Primary-treated pulp mill effluent

Two of the chief obstacles in the bioassay-guided fractionation of pulp mill effluents are yield and chemical complexity. Water samples are difficult to dry quickly, and so I explored spray drying primary-treated pulp mill effluent. Spray dried preparations generally provides a more comprehensive recovery of all compounds compared to solvent extraction which selectively removes certain classes of chemicals. After optimization of the method, I was able to completely remove the water at a rate of 1 litre every 25 hours, yielding 8 g of material from which to perform a solvent series extraction. To my knowledge, this is the first time pulp mill effluent samples have been prepared by spray drying followed by a solvent series extraction. Upon reconstitution of the spray dried material and solvent series extractions, I obtained yields between 7 – 9 times higher than comparable fractions prepared by classic solvent series or polyphenolic extraction of primary effluents (Table 5.3)(Basu et al 2009).

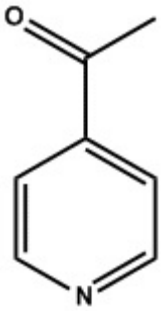


Figure 5.5 - Structure of 4-acetylpyridine, a novel compound described in pulp mill effluents.

The medium polar ethylacetate extract of the primary effluent was most active (37.3% inhibition; Fig. 5.1). This activity is comparable to ethylacetate extracts of balsam fir which I described in Chapter 4 (30% inhibition)(Waye et al 2014a). Balsam fir comprises 85% of the feedstock for this mill. I chose this ethylacetate primary effluent extract for bioassay-guided fractionation since it was the most active extract and had adequate yield for further fractionation.

Of the 11 fractions generated from the ethylacetate extract of primary effluent, fraction 9 was the most active (Fig. 5.2). Since no resin acids were present, other GAD unidentified inhibitors were responsible for inhibition and the chemical complexity of these samples indicated that the bioactivity could not be attributed to a highly-concentrated single compound, but to a complex mixture of active principles. I chose to begin my work with primary effluent in the hope that the greater extract yields typical of primary effluents compared to secondary effluents would allow us a more successful bioassay-guided

fractionation, but the yields and chemical complexity of the generated fractions were such that further fractionation was not feasible and hence not pursued. Furthermore, primary effluents are not what wild fish would be exposed to in the current environment, so I turned my efforts to work with secondary effluents.

5.4.2 Secondary-treated pulp mill effluent

Reverse osmosis concentration of secondary effluent prior to solvent series extraction resulted in reduced yields (Table 5.4) when compared to classic solvent series (5 – 70 times reduction) and polyphenolic extraction (15 – 51 times reduction) yields. The advantage to reverse osmosis concentration, however, is that larger volumes can be processed more quickly, resulting in more extract generated in absolute terms in a given time.

Like the ethylacetate extracts of balsam fir (Chapter 4; Waye et al 2014a) and primary effluent (Fig. 5.1), the ethylacetate extracts of the secondary effluent were also the most inhibitory to GAD (Fig. 5.3). These data indicate that the active constituents of balsam fir might be retained throughout the pulping process and survive secondary effluent treatment. In addition, since these active effluent extracts are highly complex and any one chemical would represent a very small part of the total extract, I predict that GAD inhibition is caused by a complex mixture of components acting in an additive or synergistic way, rather than just one compound in the mixture being responsible for the majority of the observed activity.

Of the three metabolites identified by discriminant analysis as unique to active secondary extracts, I was able to definitively confirm the identity of 4-acetylpyridine (Fig. 5.5; Table 5.5) as metabolite A in Fig. 5.4a. When injected in mice, this compound can reduce vertical climbing motor function (Bose & Pinsky 1987, reported in Hall et al 1988), has anticonvulsant properties (Pinsky & Bose 1987 reported in Hall et al 1988), and can reduce stress-induced gastric ulcers (Hall et al 1988), indicating that 4-acetylpyridine may be anti-excitory in nature in the context of neurotransmission and stress. While follow-up studies do not appear to have been performed, Hall et al (1988) allude to the similarities of these biological endpoints to those of GABAergic modulating drugs such as the benzodiazepines. From a search of the literature (ChemSpider and SciFinder) and taking into account what is known of alkaloid natural product biosynthesis (Dewick 2009), it appears that 4-acetylpyridine is non-natural in origin and may be a chemical by-product of the

pulping or secondary treatment process. This molecule was inactive at 1 mM in the GAD assay, but if an important bioactivity is identified in future work with this molecule, screening extracts of all of the wood feedstocks, of the various pulping waste streams, and the primary effluent may be warranted to help elucidate its origin. To determine if 4-acetylpyridine is natural or synthetic in nature, analysis of carbon isotopes by accelerated mass spectrometry can be performed.

Compound B and C could tentatively be identified as a methoxy and other benzoate derivatives (Fig. 5.4b; Table 5.5), but were not confirmed through co-analysis with the available purchased standards. These types of structures are consistent with those of plant origin as benzoic acids and benzoates are commonly used in the biosynthesis of many classes of secondary metabolites. Future work should include the acquisition and/or synthesis of the remaining two unique metabolites identified in active extracts that I was unable to acquire from our natural product collection or commercially (Table 5.5) in order to confirm or refute their identity. I have proposed molecular formulae for these two putative actives (Table 5.5) and I predict these unknown metabolites are isomers of those I did screen for with the same exact mass.

Presently, one of the challenges in using metabolomic techniques to analyse samples of plant origin is the limited mass spectrometry data for phytochemicals in chemical databases. As more of these phytochemicals are characterized and results are validated using MS technologies, the strength of metabolomics as a tool for phytochemical research will grow. Present NMR technology also limits the identification of isolated bioactives as the amounts of raw effluent and effort required to purify enough of the compound(s) of interest (usually around 1-2 mg) for structure elucidation would likely be prohibitive.

When I screened the same secondary extracts for several of the phytochemicals described in Chapter 4 (Waye et al 2014a) by targeted analysis, I observed the presence of the resin acids abietic acid and dehydroabietic acid in the hexane extracts and vanillin in the ethylacetate extracts. Dehydroabietic and abietic acid were both inhibitory to *in vitro* GAD, with 52% and 98% inhibition respectively, while vanillin inhibited MAO by 49% but did not inhibit GAD (Chapter 4)(Waye et al 2014a), and have I confirmed their presence in secondary effluent despite their low concentration and co-elution with other compounds by matching retention times, exact mass, elemental composition, isotopic fit, and fragmentation

pattern with standards. Resin acids can be found in effluents when mill operating conditions are sub-optimal and they can bioconcentrate in the brains of fish (reviewed in Chapter 4). The activity in my secondary extract samples was not found in the hexane extracts where the resin acids were detected, but was instead discovered in the first two ethylacetate extracts, indicating that non-resin acid compounds are inhibiting the GAD enzyme. Furthermore, I qualitatively observed that the identified resin acids represented a very small fraction of the compounds detected in the hexane fractions, which would explain why I did not see significant inhibition of GAD by the hexane fractions despite having identified these GAD inhibitors within them.

5.5 Conclusion

In this study, I have attempted two novel approaches in preparing chemical extracts of pulp effluent, combining techniques such as spray drying and reverse osmosis with more conventional solvent series extractions in the attempt to increase extract yields. Spray drying samples before extraction significantly improves yield, but is time-consuming. Reverse-osmosis before extraction, on the other hand, decreases yield on a per litre basis, but improves the absolute yield by allowing concentration of large volumes of effluent quickly.

I have also demonstrated that by using advanced techniques in analytical chemistry with UPLC-QTOF and MassLynx, significant information about the chemical composition of active fractions of these complex and dilute samples can be obtained. Using discriminant analysis, I identified a novel chemical marker, 4-acetylpyridine, in secondary treated pulp mill effluents; a compound that while not active in the GAD assay, has previously reported bioactivities that could potentially implicate the GABAergic system that modulates fish reproduction. Furthermore, using targeted analysis, I was able to detect resin acids that were previously described as neuroactive (reviewed in Chapter 4) and as active in the GAD assay in Chapter 4. Vanillin was also detected and was active in the MOA assay (Chapter 4). I was able to detect these compounds by using UPLC-QTOF despite their low concentration in effluents and other compounds that were co-eluting with them. This finding may have biological and environmental significance in fish chronically exposed to effluent due to the bioconcentration of resin acids in neural tissues and their strong *in vitro* GAD inhibitory action.

Chapter 6: Bioassay-guided fractionation of a pulp and paper mill feedstock, balsam fir, *Abies balsamea* L.Mill. (Pinaceae) and metabolomic comparison of glutamic acid decarboxylase inhibitory feedstock and effluent extracts

[1]Andrew Waye, [1]Ammar Saleem, [1]Jose-Antonio Guerrero-Analco, [1]Matthew Taylor, [1]Vance L Trudeau, [1]John T Arnason

[1]Department of Biology, University of Ottawa, Ottawa, ON, K1N 6N5

To avoid the challenges of working with highly dilute and chemically complex pulp mill effluents, the identification of phytochemical GAD inhibitors was undertaken by performing bioassay-guided fractionations of the wood feedstock, balsam fir. Comparative metabolomic techniques were applied to establish that phytochemicals from wood feedstocks survive in-mill pulping processes and effluent treatment strategies.

Statement of author contributions

AW, VLT, and JTA conceived and designed this study. Preparation of samples was performed by MT, JAGA, and AW. Chemical analyses were performed by AW, AS, and JAGA. MT and AW performed bioassays. AW and MT performed data analyses and AW prepared the manuscript with contributions from MT, VLT, and JTA.

6.1 Introduction

In Chapter 5, I demonstrated that medium polar ethylacetate extracts of primary and secondary pulp mill effluents from a thermomechanical pulp (TMP) mill that inhibited fish reproduction also contained active principles that inhibit glutamic acid decarboxylase enzyme activity (GAD). Effluents from this particular mill have long been associated with the rapid inhibition of spawning in the 5-day fathead minnow spawning assays used at FPInnovations labs (Pointe-Claire, QC, Canada). Neuroendocrine disruption of the reproductive axis could be a mechanism by which these rapid and reversible effects may occur (Basu et al 2009, Waye & Trudeau 2011). The enzyme GAD is responsible for the synthesis of γ-aminobutyric acid (GABA). In the neuroendocrine system of fish, GABA is a stimulator of luteinizing hormone (LH), and thus an important regulator of the reproductive axis (Popesku et al 2008, Trudeau 1997).

During my work with extracts and fractions of effluent samples from this TMP mill (Chapter 5), I was unable to isolate and identify pure compounds with high activity responsible for observed GAD inhibition. The active principles in the studied effluent were highly dilute, extraction efficiencies were low, and the extracts were chemically complex in nature. In Chapter 4 (Waye et al 2014a), where I screened pulp and paper conifer feedstocks, I observed a similar level of inhibition of GAD in an ethylacetate extract of balsam fir, *Abies balsamea* L. Mill. (Pinaceae), as those observed in the ethylacetate extracts of the TMP effluent (Chapter 5) where balsam fir is the primary feedstock. Here, I have attempted to isolate and identify active principles in balsam fir using bioassay-guided fractionation as a complementary approach to examining the GAD inhibitory effluent in Chapter 5. The shift in focus to wood extracts of this mill's primary feedstock is a way to identify neuroactive phytochemicals entering the mill in an abundant and less complex material.

Many phytochemicals are present in pulp mill effluents and various waste streams from pulp mills, and many of these phytochemicals have neuroactivities in receptor and enzyme systems important in the neuroendocrine control of reproduction in fish (Chapter 4). I hypothesize that neuroactive plant chemicals that are present in final effluents contribute or are responsible for the reported neuroactivities in pulp mill effluents, particularly for GAD inhibition. I have found GAD inhibition in ethylacetate extracts representing compounds that are: entering the mill (balsam fir in Chapter 4); present after processing and creation of wood

into pulp (primary effluents in Chapter 5); and in the treated water that is discharged to the environment (secondary effluents in Chapter 5). I propose that the identification of GAD inhibitors in the pulp feedstock balsam fir could provide candidate phytochemicals to screen for by targeted analysis of complex mill effluents that use balsam fir to make pulp. To support the hypothesis that neuroactive chemicals are coming from the plants used at these mills, in this study I compare the balsam fir and pulp mill GAD-inhibitory ethylacetate extracts using advanced metabolomics techniques with ultra-high-performance liquid chromatography – time-of-flight mass-spectrometry (UPLC-QTOF). Ultra-high-performance liquid chromatography provides the high resolution and rapid separation of compounds, time-of-flight mass-spectrometry allows high resolution mass measurements and the identification of predicted elemental compositions, and the comparison of fragmentation patterns and isotopic ratios allow for a thorough comparison of any compounds that are present in both effluent and feedstock extracts.

6.2 Methods

6.2.1 Balsam fir collection

Balsam fir (*Abies balsamea* (L.) Mill.; voucher number 19980 at the University of Ottawa herbarium, Ottawa, ON, Canada) was collected in a forest near Denholm, QC, Canada, in 2008 and was identified according to Scoggan (1978).

6.2.2 Balsam fir wood preparation and extraction

Bark was removed manually from the wood by peeling with a knife and the wood was chipped into shavings approximately 1cm x 5cm x 0.25cm pieces in a 6” jointer (General MFG Co. Ltd., Drummondville, QC, Canada). Balsam fir wood shavings were then air dried for 96 hours and ground into 10 kg of sawdust using a Thomas Model 4 Wiley grinding mill (mesh size 2mm; Thomas Scientific, Swedesboro, NJ, USA).

Sawdust was extracted in 1 kg batches using 10 L of ethylacetate for approximately 72 hours under agitation using a Caframo BDC 3030 stirrer (800 rpm; Caframo, Wiarton, ON, CAN). The first 3 kg were extracted three times each and the remaining 7kg were extracted once, since the yield of the second and third extractions were greatly reduced. Extracts were vacuum filtered using Whatman 1 filter paper (pore size 11 μm, diameter 11

cm, Fisher Scientific, Ottawa, ON, Canada) and roto-evaporated to dryness at 40°C at 950 mmHg, combined, and stored at 4°C until fractionation. Total combined extract yield was 83.95g (0.83% extraction efficiency) and 2 g was kept for reference material.

6.2.3 Balsam fir extract silica gel fractionation

Silica gel (1.75 kg) in hexane was loaded and packed in a glass 2.0 L open air column. Extract (81.95 g) was adsorbed to 200 g of silica and loaded into the column. The solvent regime for the mobile phase consisted of 2 L each of hexane, ethylacetate, and methanol in increasingly polar ratios. The fractions were collected in 200 ml volumes, transferred into vials, and dried under air. All eluates collected from the column were analyzed by thin layer chromatography (TLC) on silica gel and eluted by an appropriate mixture of hexane-ethylacetate, ethylacetate, or ethylacetate-methanol to allow proper chromatographic separation. Phytochemical band patterns were visualized using a UV lamp (short λ = 254 nm, long λ = 325 nm) and developed with ceric sulfate solution (5% of H_2SO_4) for oxidization. Eluates with similar banding patterns were combined to generate a total of 28 chemically distinct pooled fractions. When precipitates formed in a fraction, the fraction was separated into liquor and precipitate sub-fractions.

6.2.4 Glutamic acid decarboxylase (GAD) enzyme assay

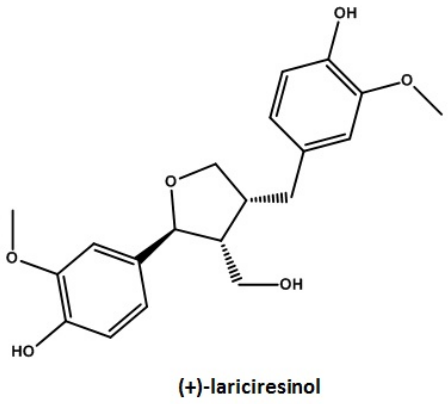
The GAD assay was performed using previously published methods (Awad et al 2009, Waye et al 2014) modified from Snedden et al (1996). Briefly, male and female goldfish (*Carassius auratus*; Aleong's International Inc., Mississauga, ON, Canada) were anesthetized with MS-222 (Syndel Laboratories, Qualicum, BC, Canada), decapitated, and whole brains were dissected, combined, and stored at -80 °C. Whole goldfish brains (roughly 20) were thawed and then homogenized in 1:10 (w/v) 10 mM phosphate buffer solution (pH 7.5) and stored in 1 ml aliquots at -80 °C. The concentration of protein in all samples was determined using the method of Bradford (1976).

To measure GAD enzyme activity, 50 µg of brain homogenate was re-suspended in glass tubes to a final volume of 350 µl of 10 mM phosphate buffer (including 60 µM pyridoxal-5'-phosphate and 120 µM dithiothreitol, pH 7.4). The reaction was initiated by addition of 30 mM glutamate containing 0.1 µCi L-[1-^{14}C] glutamic acid (50.0 mCi/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO, USA) and a suspended Whatman

GF/B filter paper soaked in Scintigest (Fisher Scientific, Ottawa, ON, Canada). Following a 45 min reaction at 37 °C, the reaction was terminated by the addition of 0.5 ml of 0.25 M HCl. The vials were allowed to incubate for another 60 min and the radioactivity (released CO_2) trapped on the filter paper was determined. All fractions were tested in triplicate at a final concentration of 50 µg/ml, (+)-lariciresinol at 1 mM, and the GAD inhibitor 3-mercaptopropionic acid (1 mM) was used as a positive control. Statistical differences ($p < 0.05$) in activities were detected using 1-way ANOVA, normality, and Levene's test in SPSS (IBM, Armonk, NY, USA) with a Tukey post-hoc test.

6.2.5 Preparative-scale HPLC

The isolation conditions for preparative-scale HPLC (prep-HPLC) were optimized on an Agilent 1100 series HPLC-DAD system and up-scaled on a 1200 series prep-HPLC-DAD system (Agilent Technologies, Santa Ana, CA, USA), equipped with a binary pump (flow rate range 5-100 ml/min), an autosampler with a 2 ml loop, a diode array detector with a flow cell (3 mm path length, maximum pressure limit 120 bar), and a fraction collector (40 ml collection tubes). The separations were performed on a reversed-phase Gemini Axia 250 x 21.2 mm column, particle size 10 µm (Phenomenex Inc., Torrance, CA). The optimal gradient conditions were 0-15 min 40-100% B at a flow rate of 31.5 ml/min and column at room temperature. Fraction F20 was selected for sub-fractionation and was fractionated into 6 phytochemically distinct sub-fractions. Of these, sub-fraction F20-4 was further fractionated into 6 sub-sub-fractions.



(+)-lariciresinol

Figure 6.1 - Chemical structure for the lignan (+)-lariciresinol, isolated from balsam fir fraction F21(P) and observed in sub-fraction F21-2.

6.2.6 Isolation and identification of (+)-lariciresinol

A precipitate was crystalized, isolated, and identified as (+)-lariciresinol (Fig. 6.1) from fraction F21 and 10 mg of precipitate in 0.6 ml of deuterated solvent was analyzed directly by nuclear magnetic resonance spectroscopy (NMR). NMR spectra were obtained from a Bruker Avance 400 MHz spectrometer (Bruker, Biospin Corporation, Billerica, MA, USA) in CD_3OD and $CDCl_3$, either at 400 MHz (^1H) or 100 MHz (^{13}C) respectively, using

tetramethylsilane as an internal standard and identification was made by comparison to spectra in Duh et al (1986).

6.2.7 UPLC-QTOF metabolomics analysis

For UPLC-QTOF analysis, separation of analytes was achieved using an Acquity BEH C18 1.7um 2.1 x 100mm column (Waters Corporation, Milford, MA) connected with a VanGuard Pre-column 2.1 x 5mm (Waters Corporation, Milford, MA). Column temperature, 50°C, sample temperature 10°C. Mobile phases were, A, water + 0.1% formic acid, B, acetonitrile + 0.1% formic acid (Fisher Optima LC-MS, Fisher Scientific Company, Ottawa, ON) with a flow rate of 0.5 ml/min. Mobile phase composition, 0-1 min 5% A isocratic, 1-6 min linear gradient 5-50% B, 6-8 min 50-95% B, 8.01-10 min 5% A isocratic (total run time 10 min). Sample injection conditions: 1uL injection followed by a strong wash 200 uL (90% acetonitrile + 10% water) and weak wash 600uL (10% acetonitrile + 90% water). QTOF analysis conditions: MassLynx software, MSe ESI+ mode, lock mass Leucine Enkephalin 12C 556.2615, source temperature 120°C, desolvation temperature 400°C, Cone gas (N_2) flow 50 L/hr, desolvation gas (N_2) flow 1195 L/hr. MSe conditions, mass range 100-1500 Da, F1 CE, 6V, F2 CER 10-30V, cone voltage 20V, scan time 1 sec., calibration 50-1000 Da sodium formate. Putative compound identifications were undertaken by comparing elemental composition and isotopic fit of target compounds using MassLynx software and finding matches in METLIN Metabolomics Database (Scripps Research Institute, San Diego, USA) and ChemSpider (Royal Society of Chemistry, London, UK). Mass fragmentation was carried out using high energy spectrum to confirm the identification using MassLynx software. For comparison of the inhibitory balsam fir fraction BF-21L with the inhibitory secondary TMP effluent samples E1 and E2 (from Chapter 4), peaks in the chromatograms with identical retention times were compared for identical mass, elemental composition, and isotopic fit.

6.3 Results

6.3.1 Bioassay-guided fractionation of balsam fir using glutamic acid decarboxylase inhibition

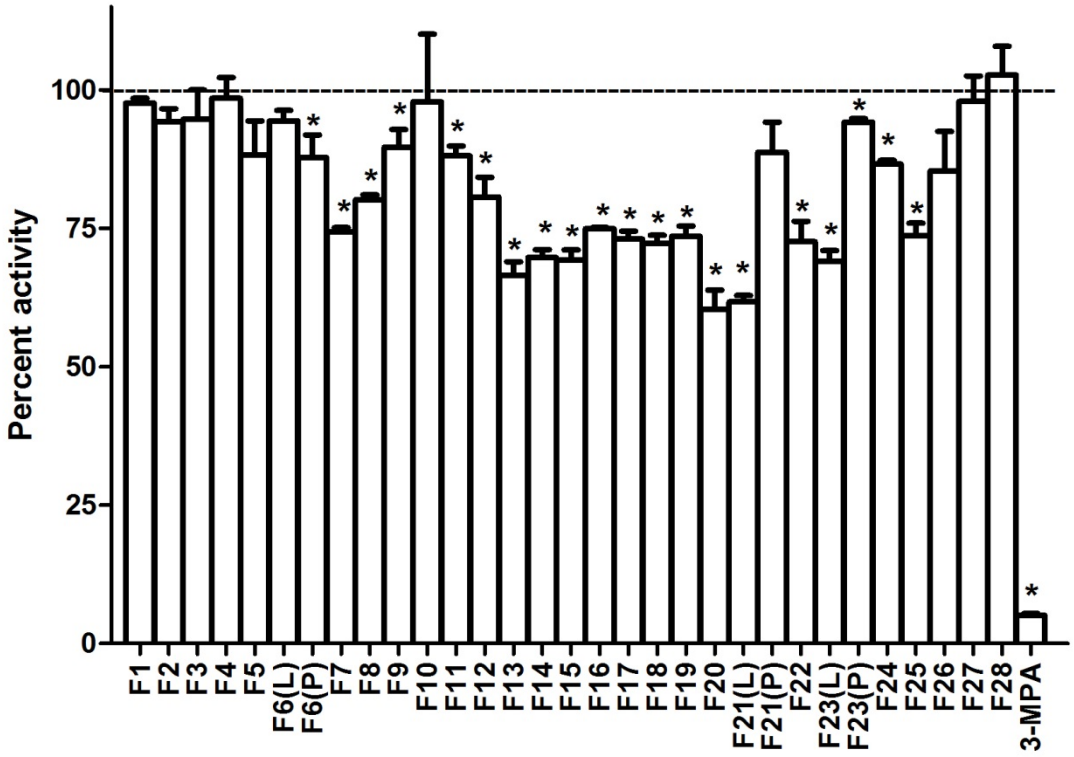


Figure 6.2 - Effects of balsam fir ethylacetate fractions on in vitro GAD activity expressed as mean percent activity of solvent controls. Error bars represent one standard error. Extracts tested in triplicate with final concentration of 50 µg/ml. Asterisk denotes significant difference from controls ($p < 0.05$). Dotted line denotes solvent control activity (100%). Fractions F6, F21, and F23 were separated into liquor (L) and precipitate (P) fractions. 3-MPA, 3-mercaptopropionic acid was tested as a positive control at 1 mM.

The yield of extraction from 10 kg of dried balsam fir was 83.95g. This extract was fractionated into 28 phytochemically distinct fractions by column chromatography. Three fractions, F6, F21, and F23, could be separated into liquor and precipitated components. Precipitate F21(P) had sufficient yield and purity to be identified as (+)-lariciresinol (Fig. 6.1) by NMR (Duh et al 1986). The resulting 31 fractions and precipitates were tested for GAD inhibition (Fig. 6.2). Of these, 20 of the 31 tested samples were inhibitory to GAD

compared to vehicle controls, but (+)-lariciresinol was inactive at the concentration of 1 mM used to screen other phytochemicals in Chapter 4 (data not shown). Fraction F20 was the most inhibitory of these (39.7% inhibition) and had a sufficient yield (322 mg) for sub-fractionation. The resulting 6 sub-fractionations (Fig. 6.3) all demonstrated GAD inhibition (16 – 50% inhibition). Upon analysis, (+)-lariciresinol was also identified as the major peak in sub-fraction F20-2. Sub- fraction F20-4 had the highest yield (66 mg) as well as demonstrated 45% GAD inhibition. Hence it was chosen for further sub-fractionation into 6 sub-sub-fractions. Sub-sub-fractions F20-4-2 (26% inhibition), F20-4-3 (17% inhibition), and F20-4-4 (23% inhibition) were inhibitory to GAD (Fig. 6.4), but to a lesser extent to the parent sub-fraction F20-4. The chemical complexity of these inhibitory sub-sub-fractions remained too high upon analysis and the yields were too low to warrant further fractionation steps.

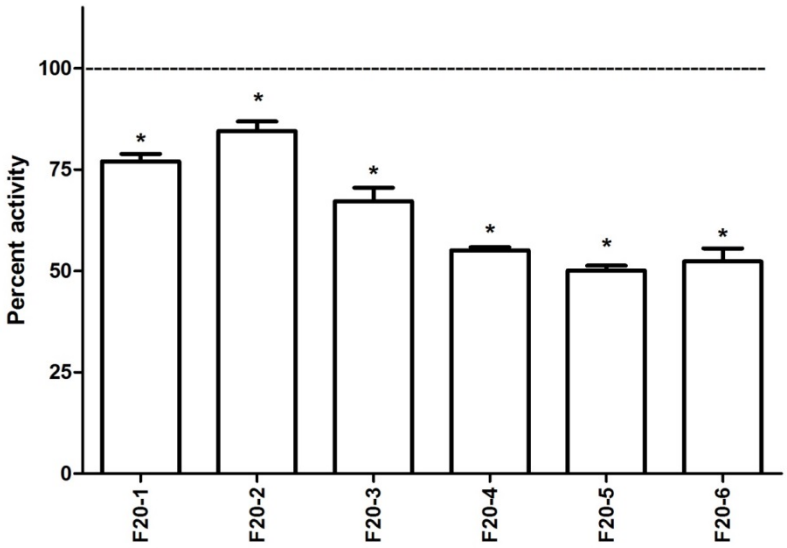


Figure 6.3 - Effects of sub-fractions of balsam fir fraction F20 on in vitro GAD activity expressed as mean percent activity of solvent controls. Error bars represent one standard error. Extracts tested in triplicate with final concentration of 50 µg/ml. Asterisk denotes significant difference from controls ($p < 0.05$). Dotted line denotes solvent control activity (100%).

6.3.2 Metabolomic analysis of active fractions

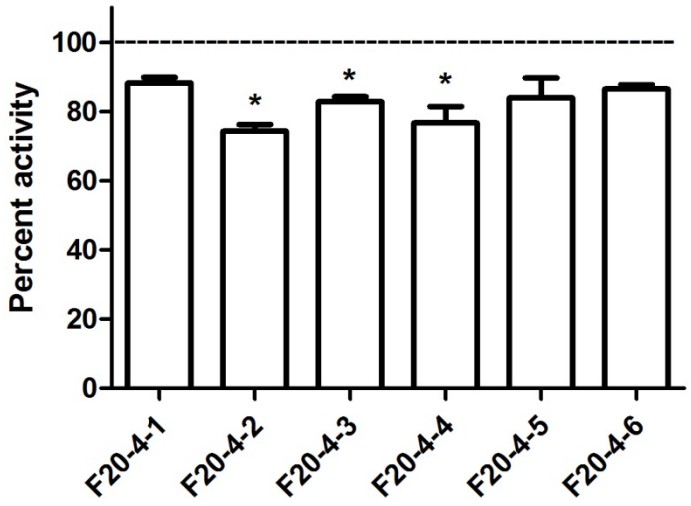


Figure 6.4 - Effects of sub-fractions of balsam fir sub-fraction F20-4 on in vitro GAD activity expressed as mean percent activity of solvent controls. Error bars represent one standard error. Extracts tested in triplicate with final concentration of 50 µg/ml. Asterisk denotes significant difference from controls ($p < 0.05$). Dotted line denotes solvent control activity (100%).

Balsam fir fraction F21(L) was chosen for advanced metabolomics analysis in positive ionization mode as it had adequate yield as well as high GAD inhibition (38% inhibition; Fig. 6.2) compared to other balsam fir fractions. Using MassLynx to assign putative chemical formulae and chemical identifications to major fraction metabolites, we tentatively identified two of the phytochemicals as N-acetylserotonin and 6-hydroxymelatonin (with monoisotopic masses of 218.1055 and 248.1161 and chemical formulae of $C_{12}H_{14}N_2O_2$ and $C_{13}H_{16}N_2O_3$, respectively). N-acetylserotonin is the precursor to melatonin in its biosynthesis from serotonin while 6-hydroxymelatonin is a metabolite of melatonin (reviewed in Krishnaswamy et al 2004). I wanted to confirm the identity of these two metabolites, and potentially other neurotransmitter precursors/metabolites in the serotonin and melatonin synthetic pathways, including L-tryptophan (precursor to serotonin), melatonin, and serotonin (Fig. 6.5)(Slominski et al 2008). No metabolites in the balsam fir fraction F21(L) were observed with the same accurate mass as L-tryptophan, melatonin, and serotonin. Metabolites with the same accurate mass as N-acetylserotonin and 6-hydroxyserotonin were indeed observed, but the UPLC retention times were markedly different (> 10s). The other relatively abundant metabolites in F21(L) putatively identified

by MassLynx include cortolone (366.4917 Da), 5-(p-aminophenyl)-1,2,3,4-tetrahydroxypentane (227.1160 Da), and N,N-dimethyl-1-(10H-phenothiazin-10-yl)-2-propanamine hydrochloride (320.1114 Da). None of these putatively identified compounds were observed in the active effluent fractions E1 and E2.

When I made a comparison of specific peaks that shared identical retention times between the active balsam fir fraction F21(L) and active effluent extracts E1 and E2, I identified 15 common metabolites (Table 6.1). Using MassLynx I was able to assign a chemical formula to 11 of these compounds, and putative identifications in METLIN to two of these. The first, with the experimental ionic mass of 247.1279, yielded nine possibilities in METLIN, with eight being di-peptides and one as being 2,4-diacetamido-2,4,6-trideoxy-beta-L-altropyranose. The second common metabolite, with the experimental ionic mass of 333.2120, to which I could assign putative identifications in METLIN resulted in six tri-peptides (Table 6.1). When I performed a targeted analysis of inhibitory balsam fir fractions for the GAD inhibiting resin acids and pinosylvin identified in Chapter 4, I was not able to confirm their presence.

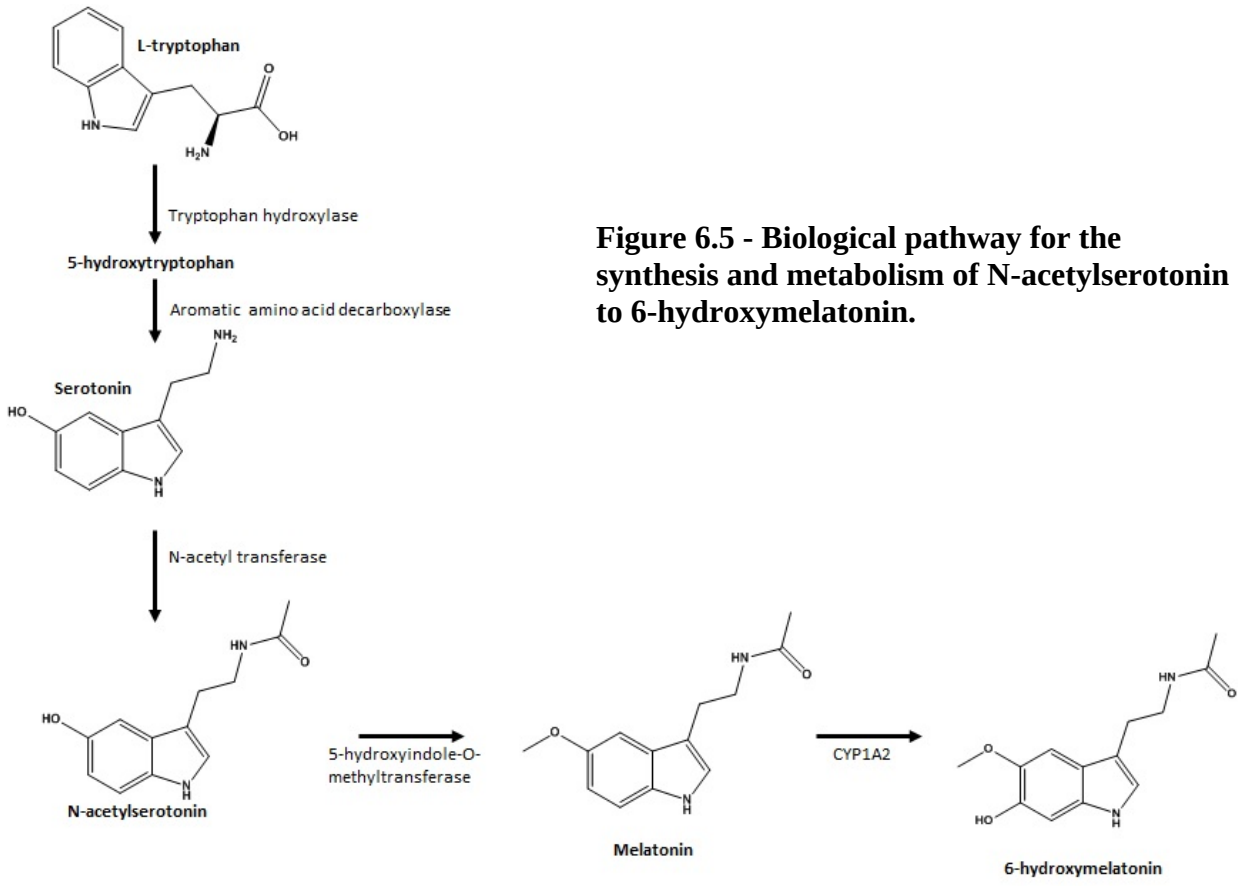


Figure 6.5 - Biological pathway for the synthesis and metabolism of N-acetylserotonin to 6-hydroxymelatonin.

6.4 Discussion

6.4.1 Bioassay-guided fractionation of balsam fir

Two of the most inhibitory fractions of the balsam fir extract were F20 and F21(L). These two fractions eluted in the non-polar to medium polarity range of the silica gel fractionation. Inhibition of GAD by medium polarity compounds agrees with our data in Chapter 5, where the ethylacetate extracts E1 and E2 had the highest inhibition. Furthermore, medium polar ethylacetate extracts of primary effluent in Chapter 5 and balsam fir in Chapter 4 were also observed to have high GAD inhibitory activity. What I have continued to observe in this chapter agrees with all other GAD data on this effluent; that medium polar extracts of this mill's primary and secondary effluent, as well as medium polar extracts of this mill's major feedstock balsam fir, contain GAD inhibitors.

Upon sequential sub-fractionation of F20 and sub-sub-fractionation of F20-4, no GAD inhibitors were isolated for identification. Despite extracting 10 kg of balsam fir wood, only 83.95 g of extract was generated with which to work with. Once two rounds of sub-fractionation were complete, the sub-fractions of BF20-4 still contained high complexity with no clear dominant peaks upon chromatographic inspection, indicating that further fractionations would not yield single compounds in sufficient quantities for NMR identification. The decrease of activity observed upon sub-fractionation of sub-fraction BF20-4 indicates that many phytochemicals may be responsible for GAD inhibition and can act together in parent fractions in an additive or synergistic fashion. The presence of a dominant, single neuroactive GAD inhibitor can be ruled out.

I was able to isolate and identify the lignan (+)-lariciresinol (Fig. 6.1) from the balsam fir fractionation, and while this lignan was not active in the GAD enzyme assay, it has other reported activities of interest. First described as a cytotoxic phytochemical in *Wikstroemia elliptica* in the *in vitro* P-388 lymphocytic leukemia assay, it has since demonstrated additional *in vitro* anticancer and anti-inflammatory (Kim et al 2011) as well as CYP3A4 inhibitory properties (Tezuka et al 2010).

Table 6.1. Phytochemical metabolites common to balsam fir fraction F21(L) and effluent fractions E1 and E2 (Chapter 5) using identical retention time and accurate mass (<5 ppm). Reported common metabolites had similar fragmentation patterns and isotopic ratios.

Retention time (min)	Accurate mass + H (Da)	Elemental composition	Putative identification
2.834	247.1279	$C_{10}H_{18}N_2O_5$	Leu-Asp Asp-Leu Ile-Asp Asp-Ile Glu-Val Val-Glu L-γ-glutamyl-L-valine L-β-aspartyl-L-leucine 2,4-bis(acetamido)-2,4,6-trideoxy-β-L-altropyranose
3.413	333.2120	$C_{14}H_{29}N_4O_5$	Lys-Val-Ser Lys-Ser-Val Val-Ser-Lys Val-Lys-Ser Ser-Val-Lys Ser-Lys-Val
5.318	397.3719	$C_{25}H_{49}O_3$	--
5.410	357.2986	$C_{23}H_{37}N_2O$	--
5.410	339.2972	$C_{20}H_{39}N_2O_2$	--
5.410	379.3154	$C_{26}H_{39}N_2$	--
5.410	393.3414	$C_{19}H_{45}N_4O_4$	--
5.410	265.2452	$C_{13}H_{33}N_2O_3$	--
5.410	247.2192	$C_{14}H_{31}O_3$	--
5.410	111.0397	--	--
5.410	124.0559	--	--
5.806	455.3753	$C_{26}H_{51}N_2O_4$	--
5.806	124.0555	$C_2H_{10}NO_4$	--
5.806	165.9958	--	--
5.806	139.9636	--	--

Leu – leucine, Asp – aspartic acid, Ile – isoleucine, Glu – glutamic acid, Val – valine, Lys – lysine, Ser – serine. Metabolites to which elemental compositions or tentative identifications could be assigned are denoted by hashes (--).

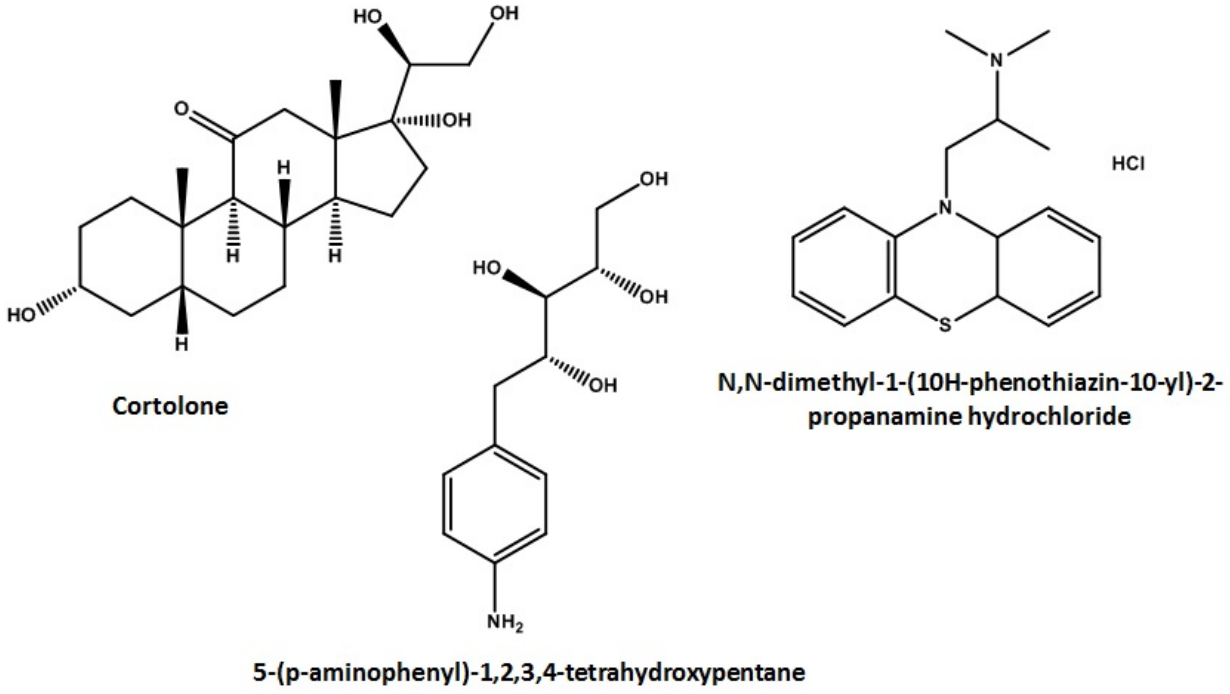
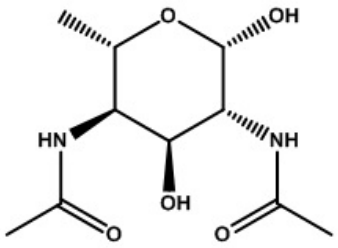


Figure 6.6 - Three tentative identifications of abundant metabolites by metabolomic analysis of balsam fir fraction F21(L).

6.4.2 Metabolomic analysis of active fractions

Using MassLynx software, I was able to identify two of the abundant metabolites in balsam fir fraction F21(L) as isomers of N-acetylserotonin and 6-hydroxymelatonin. Upon searching in the Chemspider database for the molecular formula of N-acetylserotonin, $C_{12}H_{14}N_2O_2$, 2048 compounds were found, indicating that identification of which specific isomer of $C_{12}H_{14}N_2O_2$ is present in the sample may pose challenges. Upon searching for the elemental composition of 6-hydroxymelatonin, $C_{13}H_{16}N_2O_3$, 2257 isomers were identified. Of the three other abundant metabolites putatively identified in F21(L), cortolone (Fig. 6.6), given its similar structural characteristics, may be a phytosterol metabolite. In the literature, cortolone has not been described in plants, but in animals it is a steroid metabolite that is eventually glucuronidated and excreted in urine (Jerjes et al 2006). The only phytochemical reference I was able to find on the molecular formula of cortolone ($C_{21}H_{34}O_5$) is shahamin B, described in jambul tree (*Syzygium cumini* L.) seeds (Banerjee & Narendhirakannan 2011). The tentatively identified 5-(p-aminophenyl)-1,2,3,4-tetrahydroxypentane does not appear to be phytochemical in nature (Fig 6.6) and has no reported bioactivities through searches of

Chemspider and PubChem databases. The third tentative identification, N,N-dimethyl-1-(10H-phenothiazin-10-yl)-2-propanamine hydrochloride (Fig. 6.6), is a pharmaceutical used as an antihistamine, antiemetic, and sedative. Also called promethazine, it is a histamine receptor 1, muscarinic receptor 1, and dopamine receptor 2 antagonist (Page et al 2009) but is unlikely to be phytochemical in nature given the structure. Since none of these metabolites were detected in the GAD inhibitory effluent extracts E1 and E2, we did not confirm their identities by purchasing standards for direct analytical comparison. To our knowledge, none of these three metabolites have been reported in plants, demonstrating a limitation to the databases for metabolomics work on plants. One of the challenges of using current metabolomics databases is the bias towards human and animal metabolites. As more phytochemical metabolites are added to the databases, future analyses and the identification of phytochemical metabolites in effluent and plant extracts will be more successful. None of the GAD inhibitors (resin acids and pinosylvin) in Chapter 4 were identified in the active balsam fir fractions.



2,4-diacetamido-2,4,6-trideoxy-beta-L-altropyranose

Figure 6.7 - Chemical structure of tentatively identified compound 2,4-diacetamido-2,4,6-trideoxy-beta-L-altropyranose, common to GAD inhibitory fractions of balsam fir F21(L) and secondary effluent fractions E1 and E2 (described in Chapter 5).

Comparison of balsam fir F21(L) with the two active extracts in Chapter 5, E1 and E2, demonstrated that chemical similarities exist between the phytochemical composition of the major feedstock species and the resulting treated effluent. Upon searching solely in areas of the chromatogram where clear peaks were identical between F21(L), E1, and E2, I was able to report 15 common metabolites. For 11 of these I could attribute chemical formula and for two I was able to propose putative structures using METLIN as di- and tri-peptide isomers or 2,4-diacetamido-2,4,6-trideoxy-beta-L-altropyranose (Table 6.1). The non-peptidic 2,4-diacetamido-2,4,6-trideoxy-beta-L-altropyranose (Fig. 6.7) is an intermediary product of pseudaminic acid synthesis. Pseudaminic acid is a sugar unique to bacteria and is

important for flagellar assembly and motility (Schoenhofen et al 2006). The putative identification of this molecule in balsam fir and mill effluents is not unexpected as its source could be from flagellated bacterial endophytes and/or the bacterial sludge used in the secondary treatment of effluents.

6.5 Conclusion

In this chapter, I was able to isolate and identify (+)-lariciresinol, a lignan that while not active for GAD inhibition, has described therapeutic potential. I was able to assign chemical formulas to five phytochemical markers unique to a GAD inhibitory balsam fir fraction, and 11 phytochemical markers common to GAD inhibitory fractions of both balsam fir and effluent extracts. The chemical similarities between balsam fir and effluent extracts observed in this analysis clearly demonstrates that tree feedstocks used at pulp and paper mills can contribute bioactive phytochemicals that can survive mill processing and effluent treatment to emerge in discharged wastewater. The results also demonstrate that there is no single dominant active principle, but activity is associated with chemically complex fractions. Here, I demonstrated that by using advanced metabolomic techniques with UPLC-QTOF, one can generate information that may facilitate the future isolation and identification of bioactive compounds from chemically complex and dilute mixtures such as pulp mill effluents.

Chapter 7: General Discussion

The research presented in this thesis is among the first to support the hypothesis that neuroendocrine disruption can explain impairment of reproduction in fish exposed to pulp mill effluents. The importance of this work is highlighted by the fact that in thirty years of research on the reproductive effects of pulp mill effluents, only one paper (Van Der Kraak et al 1992) directly measured the reproductive impacts of effluents upstream of the gonad and liver in the reproductive hormonal axis. In that study, Van Der Kraak et al (1992) found that the pituitary's ability to release LH was impaired upon stimulation by GnRH in an effluent-exposed wild population of white sucker (*Catostomus commersonii*). Earlier work from our research group directly measured neuroendocrine effects at the level of the brain, where the hypothesis was first presented that neuroendocrine disruption may be the mechanism by which reproductive endpoints in fish were being upset by exposure to effluents (Basu et al 2009, Popesku et al 2010). This novel hypothesis (H1) and the research to support it in each chapter of this thesis has provided: 1) an understanding of the mechanism (the inhibition of ovulation; Chapter 2) by which fathead minnow spawning inhibition occurs in the assay used by industry to assess effluent quality; 2) the identification of novel neuroendocrine pathways by which reproduction may be inhibited by effluents, their wood feedstocks, and phytochemicals in effluents (Chapters 3, 4, 5 and 6); 3) a list of neuroactive extracts and phytochemicals abundant in Canadian boreal forests that will be of interest in future environmental research and as potential natural product leads for the treatment of neurological disorders (Chapters 3 and 4); 4) the identification of confirmed and putative compounds never before described in pulp mill effluents and balsam fir wood from which further research can be performed (Chapters 5 and 6); and 5) the application of advanced metabolomics techniques to analyze and compare complex and dilute environmental samples (Chapters 5 and 6).

While this thesis contributes to the field of research that informs the isolation and characterization of neuroendocrine disruptors, certain limitations existed in the definitive identification of neuroendocrine disruption of reproduction of wild fish exposed to pulp mill effluents. My *in vivo* experiment in Chapter 2 was with an assay where fathead minnow were exposed to 100% effluent and no direct measure of neuroendocrine function at the level

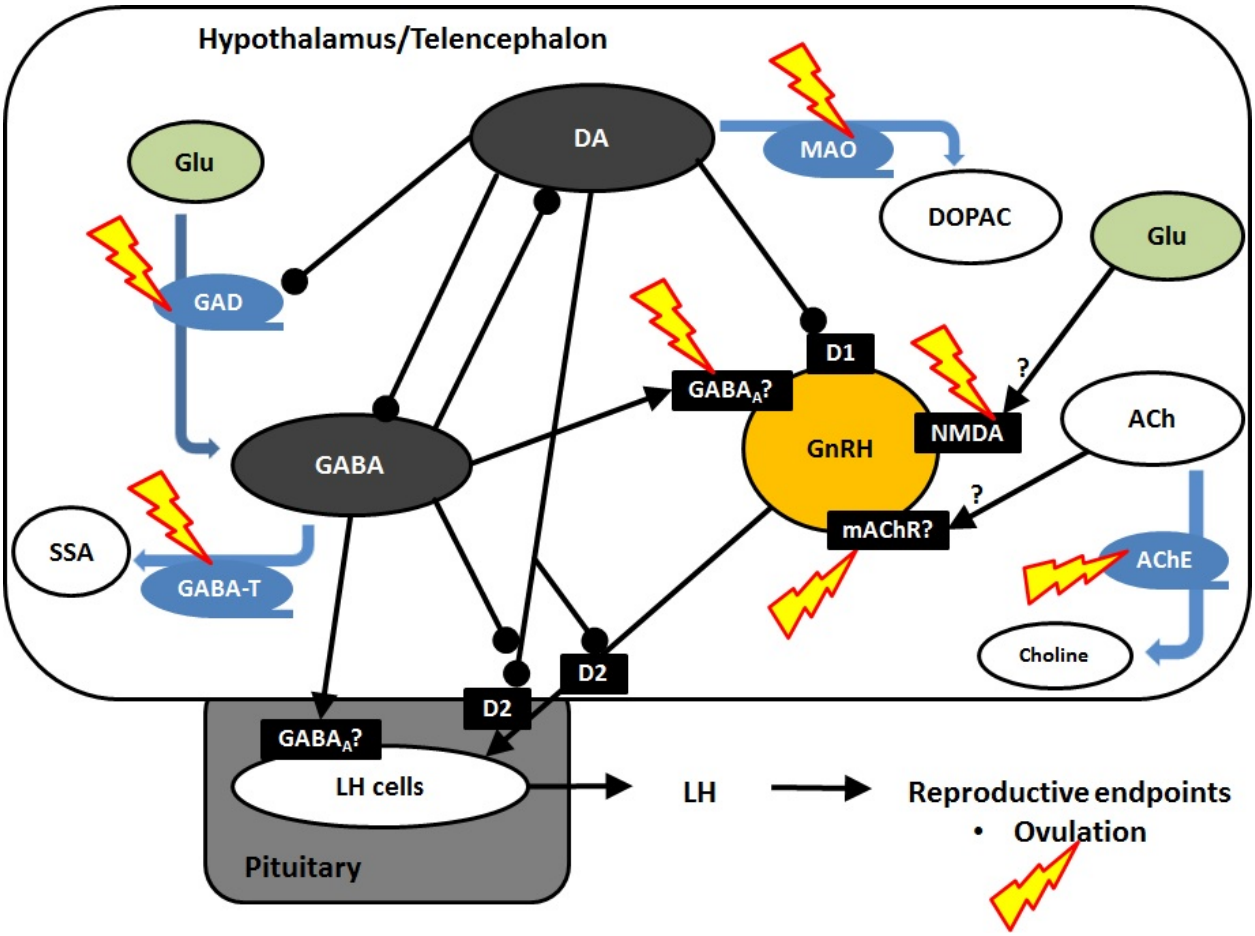


Figure 7.1 - Hypothetical model demonstrating sites where disruption of reproduction in fish may occur. Lightning bolts identify specific hypothetical neuroendocrine sites of action (with the exception of ovulation) tested in this thesis. Arrowed lines signify stimulation while circled lines signify inhibition. Black boxes represent receptors while curved arrows with attached graphics signify enzymatic reactions. Question marks exist where biological activity is not thoroughly characterized. DA, dopamine; MAO, monoamine oxidase; DOPAC, 3,4-dihydrophenylacetic acid; Glu, glutamic acid; GAD, glutamic acid decarboxylase; GABA, γ-aminobutyric acid; GABA-T, GABA transaminase; SSA, succinic semialdehyde; GABA(A), GABA type A receptor; D1, dopamine type 1 receptor; D2, dopamine type 2 receptor; GnRH, gonadotropin-releasing hormone; NMDA, N-methyl-D-aspartate receptor; mAChR, muscarinic acetylcholine receptor; ACh, acetylcholine; AChE, acetylcholinesterase; LH, luteinizing hormone.

of the brain was performed. A previous *in vivo* study by Popesku et al (2010) did measure neuroendocrine endpoints though and demonstrated that exposure of fathead minnows to reproductively inhibitory effluents elicited similar transcriptional changes as goldfish

exposed to dopamine agonists, suggesting that GnRH and LH-modulatory pathways, such as dopaminergic inhibition, are being impacted by effluents. My experiment in Chapter 2 had the potential to refute the neuroendocrine disruption hypothesis (H1), since if ovulation and milt production was observed, it would demonstrate that reproductive neuroendocrine function and its ability to stimulate an appropriate release of LH would be intact. However this was not the case. Other mechanisms exist for the reproductive effects seen in lab and wild fish exposed to effluent, such as potential effects on gonadal gonadotropic hormone receptors or by directly interacting with steroidogenic enzymes. While many effluents are anti-reproductive, they inconsistently affect steroidal pathways (Munkittrick et al. 1998, Wartman et al 2009), and it is unlikely that reproductive inhibition is simply a general stress response since there is little evidence for stress induction as measured by cortisol (Linton et al 2005, McMaster et al 2006). These inconsistencies lend support to the idea that interactions of effluents with the reproductive axis is likely complex and includes effects at not only the gonad and liver, but also at the level of the neuroendocrine brain and pituitary.

A hypothetical model showing each of the specific sites where neuroendocrine disruption could occur from exposure to effluent or feedstock extracts or from phytochemicals described in effluent waste streams is presented in Figure 7.1. A comprehensive summary of the implications of each of the measured neuroendocrine processes in this thesis are outlined in Table 7.1 according to the samples tested. Over 100 extracts and fractions and 18 compounds were tested in a combination of nine neuroendocrine assays, identifying 190 novel bioactivites. Ideally, this research would have isolated and identified a potent active principle of with specific demonstrable *in vivo* disruption of neuroendocrine pathways. This would allow researchers and industry to elucidate with more certainty novel mechanisms by which reproduction is being impacted in wild fish to allow for the selective treatment and removal of the neuroactive substance from mill effluents. The reality is that there are multiple neuroactive fractions and even fractions that have gone through several fractionations have multiple compounds that are not easily identified. The challenges encountered in this thesis while working with pulp mill effluents will still require additional research in order to achieve the long-term goal of removing all reproductive stressors from exposed fish populations and elucidating the mechanism(s) by which it occurs. This research does, however, provide evidence that neuroendocrine

pathways that control reproduction can be impacted by constituents of pulp mill effluent, and that some of this evidence is quite compelling. For example, resin acids were detected in treated effluent fractions (Chapter 5), are potent GAD inhibitors (Chapter 4), and can bioconcentrate in the brain (Oikari et al 1982).

The identification of putative neuroendocrine disruptors also has neuropharmacological significance and supports the hypothesis that constituents of effluents have the potential for development of natural health products (H2). For example, there was the promising structure-activity of MAO inhibition observed by the phytochemicals tested in Chapter 4. Many MAO inhibitors are currently being used to treat a variety of neurological disorders, thereby providing an avenue by which compounds abundant in the Canadian boreal forest may be developed into potential natural health products or leads for pharmaceutical drugs. In order to establish this potential for value-added products for the Canadian forest products industry, safety and efficacy data of these phytochemicals need to be generated in mammalian models for disorders such as anxiety, depression, Parkinson's disease, etc where MAO inhibitors are currently being used as treatment options. Pharmacokinetics and pharmacodynamics can be performed to assess derivatization to increase bioavailability and potency and to establish estimates of a human equivalent dose for future clinical studies.

In order to test the neuroendocrine disruption of reproduction (model presented in Fig. 7.1) a series of *in vivo* experiments could be performed in the 5-day fathead minnow spawning assay, where fish would be exposed to inhibitory effluents alongside co-treatments of selective dopamine and GABA agonists and antagonists (with appropriate controls) and ovulation (and milt production) and spawning success could be measured. With the HPLC-MS/MS (QTRAP) technology in the uOttawa Laboratory for the Analysis of Natural and Synthetic Environmental Toxicants (LANSET) facilities, advanced quantitative analyses of specific neurotransmitter levels responsible for controlling the reproductive axis in the neuroendocrine brain (hypothalamus and telencephalon) and pituitary could be performed using highly selective multiple reaction monitoring (MRM). Once changes in specific neurotransmitter levels have been identified, *in vitro* activities from exposed fish of specific enzymes responsible for the synthesis and metabolism of these neurotransmitters could be

tested. Discriminant analysis of the metabolite profile of the brains of these exposed fish using UPLC-QTOF could identify exogenous compounds in effluents that are entering the brains of fish exposed to inhibitory effluents. If these are novel compounds which cannot be confirmed by co-chromatography, screening of the feedstock species of the mill for the identified putative actives in effluents would facilitate the isolation and identification (e.g., by nuclear magnetic resonance) of neuroendocrine disruptors. Once in hand, these compounds could be tested in fathead minnows to demonstrate whether or not they are indeed responsible for the reproductive inhibition seen in lab studies and to establish toxicokinetic and toxicodynamic characterization. Once tested, the(se) neuroactive molecule(s) and the specific neurotransmitter(s) impacted can then be screened in the neuroendocrine brain of wild fish populations where reproductive effects are observed using advanced and sensitive targeted analysis with HPLC-QTRAP. This would extend the lab results to wild fish populations in order to assign specific molecules to specific mechanisms of action in the environmentally-relevant context.

The work presented in this thesis is an important first step towards the identification of real-world fish populations experiencing neuroendocrine upsets to reproduction, and provides the ground-work for further elucidation of ecotoxicological effects (in various contexts) in the emerging field of neuroendocrine disruption.

Table 7.1. Hypothetical neuroendocrine reproductive implications in fish of measured *in vitro* sample neuroactivities.

	Enzymes				Receptors				
Sample	GAD	MAO	GABA-T	AchE	GABA(A)	GABA(A)-BZD	D2	mAChR	NMDA
Hardwood woods[a]									
Green ash				+				+/-	
Basswood		-		+				+/-	
Beech				+				+/-	+/-
Bitternut hickory	++	-	+	+			+/-		
Butternut	-	-		+			+/-		
Elm	-		+	+					+/-
Ironwood	+	-	+	++				+/-	+/-
Largetooth aspen	-						+/-	+/-	
Red maple				++			+/-	+/-	
Red oak		-	+	++					
Shagbark hickory				++				+/-	
Sugar maple	+	-		++				+/-	
White birch	-	-	+	+				+/-	
Yellow birch	-		-				+/-		
Hardwood barks[a]									
Green Ash	+			+					
Basswood			++	++					
Beech			+	+			+/-		
Bitternut hickory				+				+/-	
Butternut	--			+	+/-			+/-	
Elm									
Hybrid poplar	+	-	+	+				+/-	
Largetooth aspen		-	+	+				+/-	
Red maple	-	-		+			+/-	+/-	
Red oak		-	-	++					
Shagbark hickory		-	++	++			+/-		
Sugar maple				+			+/-		
White birch	+	-		+					
Yellow birch				+	+/-		+/-		

Table 7.1 (cont). Hypothetical neuroendocrine reproductive implications in fish of measured *in vitro* sample neuroactivities.

	Enzymes				Receptors				
Sample	GAD	MAO	GABA-T	AchE	GABA(A)	GABA(A)-BZD	D2	mAChR	NMDA
Conifer MeOH[b]									
Balsam fir	-	-							
Black spruce		--							
White spruce	-	--							
Tamarack	-	-							
Jack pine	-	-							
White pine	-	-							
Eastern hemlock		-							
White cedar	-	-							
Juniper		-				+/-			
Conifer EtOAc[b]									
Balsam fir	-	-							
Black spruce	-	-							
White spruce	-	--				+/-			
Tamarack	-	-							
Jack pine	-	-							
White pine	-								
Eastern hemlock	-	-							
White cedar	-	-							
Juniper		-							
Primary effluent fractions									
Hexane	-								
Ethylacetate	-								
Aqueous	-								

Table 7.1 (cont). Hypothetical neuroendocrine reproductive implications in fish of measured *in vitro* sample neuroactivities.

	Enzymes				Receptors				
Sample	GAD	MAO	GABA-T	AchE	GABA(A)	GABA(A)-BZD	D2	mAChR	NMDA
Primary sub- fractions[c]									
F1	-								
F2	-								
F3	-								
F4	-								
F5	-								
F6	-								
F7	-								
F8	-								
F9	-								
F10	-								
F11	-								
Secondary effluent fractions									
H1									
H2									
E1	-								
E2	-								
E3									
Aqueous									
Balsam fir EtOAc fractions[d]									
F1									
F2									
F3									
F4									
F5									
F6(L)									
F6(P)	-								
F7	-								
F8	-								
F9	-								

Table 7.1 (cont). Hypothetical neuroendocrine reproductive implications in fish of measured *in vitro* sample neuroactivities.

	Enzymes				Receptors				
Sample	GAD	MAO	GABA-T	AchE	GABA(A)	GABA(A)-BZD	D2	mAChR	NMDA
Balsam fir EtOAc fractions									
F10									
F11	-								
F12	-								
F13	-								
F14	-								
F15	-								
F16	-								
F17	-								
F18	-								
F19	-								
F20	-								
F21(L)	-								
F21(P)									
F22	-								
F23(L)	-								
F23(P)	-								
F24	-								
F25	-								
F26									
F27									
F28									
Balsam fir F20 sub-fractions									
F20-1	-								
F20-2	-								
F20-3	-								
F20-4	-								
F20-5	-								
F20-6	-								

Table 7.1 (cont). Hypothetical neuroendocrine reproductive implications in fish of measured *in vitro* sample neuroactivities.

	Enzymes				Receptors				
Sample	GAD	MAO	GABA-T	AchE	GABA(A)	GABA(A)-BZD	D2	mAChR	NMDA
Balsam fir F20-4 sub-sub-fractions									
F20-4-1									
F20-4-2	-								
F20-4-3	-								
F20-4-4	-								
F20-4-5									
F20-4-6									
Phytochemicals									
Juglone	-	-							
Geranyl linalool		+++							
4-ethylguaiacol		--							
Vanillin		-							
Veratraldehyde		-							
Manool									
Isoeugenol		---							
3-OH-5-MeO-stilbene	-	-							
Pinosylvin	-	+							
Dehydorabietic acid	--								
Neoabietic acid	--								
Abietic acid	---								
Levopimaric acid	--	++							
Palustric acid	--	+							
Sandaracopimaric acid	--								
Isopimaric acid	--								
4-acetylpyridine									
(+)-lariciresinol									

GAD, glutamic acid decarboxylase; MAO, monoamine oxidase; GABA-T, γ-aminobutyric acid transaminase; AChE, acetylcholine esterase; GABA(A), GABA type A receptor; GABA(A)-BZD, benzodiazepine-binding site of GABA(A); D2, dopamine type 2 receptor; mAChR, muscarinic ACh receptor; [a]80% ethanol extracts of hardwood wood or bark; [b]Methanol (MeOH) or ethylacetate (EtOAc) extracts of conifer wood; [c]Fractions of effluent EtOAc extract; +, expected stimulation of reproductive axis (++ >50% and +++ >100% of controls); -, expected inhibition of reproductive axis (-- <50% and --- <75% of controls). Black boxes denote samples were not tested in given assay.

References

Abbott E, Hall D, Hamberger B, Bohlmann J. 2010. Laser microdissection of conifer stem tissues: Isolation and analysis of high quality RNA, terpene synthase enzyme activity and terpenoid metabolites from resin ducts and cambial zone tissue of white spruce (Picea glauca). BMC Plant Biology 10: 106

Ali M, Sreekrishnan TR. 2001. Aquatic toxicity from pulp and paper mill effluents: A review. Advances in Environmental Research 5: 175-96

Ankley GT, Jensen KM, Kahl MD, Korte JJ, Makynen EA. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (Pimephales promelas). Environmental Toxicology and Chemistry 20: 1276-90

Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, et al. 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (Pimephales promelas). Toxicological Sciences 67: 121-30

Arnason T, Hebda RJ, Johns T. 1981. Use of plants for food and medicine by Native Peoples of eastern Canada. Canadian Journal of Botany 59: 2189-325

Ashton H, Young AH. 2003. GABA-ergic drugs: Exit stage left, enter stage right. Journal of Psychopharmacology 17: 174-78

Awad R, Ahmed F, Bourbonnais-Spear N, Mullally M, Ta CA, et al. 2009. Ethnopharmacology of Q'eqchi' Maya antiepileptic and anxiolytic plants: Effects on the GABAergic system. Journal of Ethnopharmacology 125: 257-64

Awad R, Arnason JT, Trudeau V, Bergeron C, Budzinski JW, et al. 2003. Phytochemical and biological analysis of Skullcap (Scutellaria lateriflora L.): A medicinal plant with anxiolytic properties. Phytomedicine: International Journal of Phytotherapy and Phytopharmacology 10: 640-49

Awad R, Levac D, Cybulska P, Merali Z, Trudeau V, et al. 2007. Effects of traditionally used anxiolytic botanicals on enzymes of the gamma-aminobutyric acid (GABA) system. Canadian Journal of Physiology and Pharmacology 85: 933 - 42

Baird-Lambert J, Davis PA, Taylor KM. 1982. Methylaplysinopsin: A natural product of marine origin with effecty on serotonergic neurotransmission. Clinical and Experimental Pharmacology and Physiology 9: 203-12

Banerji A, Poddar MK, Ghosh JJ. 1977. Action of Δ9-tetrahydrocannabinol on membrane-bound monoamine oxidase activity. Toxicology and Applied Pharmacology 40: 347-54

Barrett TJ, Lowell RB, Tingley MA, Munkittrick KR. 2010. Effects of pulp and paper mill effluent on fish: A temporal assessment of fish health across sampling cycles. Environmental Toxicology and Chemistry 29: 440-52

Basu N, Scheuhammer AM, Evans RD, O'Brien M, Chan HM. 2007. Cholinesterase and monoamine oxidase activity in relation to mercury levels in the cerebral cortex of wild river otters. Human & Experimental Toxicology 26: 213-20

Basu N, Ta CA, Waye A, Mao J, Hewitt M, et al. 2009. Pulp and paper mill effluents contain neuroactive substances that potentially disrupt neuroendocrine control of fish reproduction. Environmental Science & Technology 43: 1635-41

Basu N, Waye A, Trudeau VL, Arnason JT. 2012. Extracts from hardwood trees used in commercial paper mills contain biologically active neurochemical disruptors. Science of The Total Environment 414: 205-09

Bedon F, Bomal C, Caron S, Levasseur C, Boyle B, , et al. 2010. Subgroup 4 R2R3-MYBs in conifer trees: Gene family expansion and contribution to the isoprenoid- and flavonoid-oriented responses. Journal of Experimental Botony 61: 3847-64

Bergonzelli GE, Donnicola D, Porta N, Corthesy-Theulaz IE. 2003. Essential oils as components of a diet-based approach to management of Helicobacter infection. Antimicrobial Agents and Chemotherapy 47: 3240-6

Bicho PA, Martin V, Saddler JN. 1995. Growth, induction, and substrate specificity of dehydroabietic acid-degrading bacteria isolated from a kraft mill effluent enrichment. Applied and Environmental Microbiology 61: 3245-50

Bose R, Pinsky C. 1987. 3- and 4-acetylpyridine display differential potency on central dopaminergic function in mice. Proceedings of the Canadian Federation of Biological Societies 30: 111

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-54

Campbell MM, Ellis BE. 1992. Fungal elicitor-mediated responses in pine cell cultures: III. Purification and characterization of phenylalanine ammonia-lyase. Plant Physiology 98: 62-70.

Chang LC, Song LL, Park EJ, Luyengi L, Lee KJ, et al. 2000. Bioactive constituents of Thuja occidentalis. Journal of Natural Products 63: 1235-8

Cheng S, D'Cruz I, Wang M, Leitch M, Xu C. 2010. Highly efficient liquefaction of woody biomass in hot-compressed alcohol-water co-solvents. Energy and Fuels 24: 4659-67

Chiodera P, Volpi R, d'Amato L, Petrolini R, Salati G, et al. 1985. Muscarinic cholinergic mediation of LHRH-induced LH and FSH secretion in normal men. Clinical endocrinology 23: 361-6

Christianson-Heiska I-L, Haavisto T, Paranko J, Bergelin E, Isomaa B. 2008. Effects of the wood extractives dehydroabietic acid and betulinol on reproductive physiology of zebrafish (Danio rerio)—A two-generation study. Aquatic Toxicology 86: 388-96

Clausen TP, Reichardt PB, Bryant JP. 1986. Pinosylvin and pinosylvin methyl ether as feeding deterrents in green alder. Journal of Chemical Ecology 12: 2117-31

Clayton JB. 1997. Effects of photoperiod on the reproductive success of fathead minnows (Pimephales promelas). MSc Thesis; Texas Tech University

Colovic MB, Krstic DZ, Lazarevic-Pasti TD, Bondzic AM, Vasic VM. 2013. Acetylcholinesterase inhibitors: pharmacology and toxicology. Current neuropharmacology 11: 315-35

Conner AH, Diehl MA, Rowe JW. 1980. Tall oil precursors and turpentine in jack and eastern white pine. Wood Science 12: 194-200

Degtyarenko AS, Buinova EF, Pertsovskii AL, Izotova LV, Antonov VI, et al. 1998. Acids in products of the processing of wood greens and bark of fir and cedar. Koksnes Kimija 2: 109-11

De Marino S, Cattaneo F, Festa C, Zollo F, Iaccio A, et al. 2011. Imbricatolic acid from Juniperus communis L. prevents cell cycle progression in CaLu-6 cells. Natural Products Chemistry 77: 1822-8

Demers PA, Teschke K, Davies HW, Kennedy SM, Leung V. 2000. Exposure to dust, resin acids, and monoterpenes in softwood lumber mills. American Industrial Hygene Association Journal 61: 521-8

Demetriades AK, Wallman PD, McGuiness A, Gavalas MC. 2005. Low cost, high risk: accidental nutmeg intoxication. Emergency medicine journal 22: 223-5

De Pascual TJ, San Feliciano A, Barrero AF. 1973. Composition of Juniperus communis (common juniper) fruit. Anales de Quimica 69: 1065-7

De Pascual TJ, Barrero AF, Muriel L, San Feliciano A, Grande M. 1980. New natural diterpene acids from Juniperus communis. Phytochemistry 19: 1153-6

Dewick PM. 2009. Alkaloids. In Medicinal Natural Products. pp. 311-420. John Wiley & Sons, Ltd: Chichester, UK

Deyoe DR, Brown GN. 1979. Glycerolipid and Fatty Acid Changes in eastern white pine chloroplast lamellae during the onset of winter. Plant Physiology 64: 924-9

Duke JA. 1992. Handbook of phytochemical constituents of GRAS herbs and other economic plants. CRC Press: Boca Raton, USA

Dufour S, Sebert ME, Weltzien FA, Rousseau K, Pasqualini C. 2010. Neuroendocrine control by dopamine of teleost reproduction. Journal of Fish Biology 76: 129-60

Duh C-Y, Phoebe CH, Pezzuto JM, Kinghorn AD, Farnsworth NR. 1986. Plant anticancer agents, XLII. Cytotoxic constituents from Wikstroemia elliptica. Journal of Natural Products 49: 706-09

Eaton AD, Franson MAH. 2005. Standard Methods for the Examination of Water & Wastewater. American Public Health Association

Eberhardt TL, Han JS, Micales JA, Young RA. 1994. Decay resistance in conifer seed cones: Role of resin acids as inhibitors of decomposition by white-rot fungi. Holzforschung 48: 278-84

Erdtman H. 1943. The phenolic constituents of pine heart wood. IV. Membrane-forming substances in pine heart wood. Svensk Papperstidn 46: 226-8

Erdtman H. 1944. The phenolic constituents of pine heartwood. V. The heartwood of Pinus strobus. Svensk Kemisk Tidskrift 56: 2-14

Figueroa HR, Hall TR, Olcese JM, de Vlaming VL. 1981. Effects of inhibitors of monoamine oxidase activity in goldfish (Carassius auratus) brain in vitro. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 70: 281-84

Floody OR. 2014. Role of acetylcholine in control of sexual behavior of male and female mammals. Pharmacology Biochemistry and Behavior 120: 50-56

FPAC. 2014. Industry by the numbers. http://www.fpac.ca/index.php/en/page/industry-by-the-numbers: Forest Products Association of Canada

Gawienowski AM, Chatterjee D, Anderson PJ, Epstein DL, Grant WM. 1982. Effect of delta 9-tetrahydrocannabinol on monoamine oxidase activity in bovine eye tissues, in vitro. Investigative Ophthalmology & Visual Science 22: 482-5

Geraldo de Carvalho M, Cranchi DC, Geraldo de Carvalho A. 1996. Chemical constituents from Pinus strobus var. chiapensis. Journal of the Brazilian Chemical Society 7: 187-91

Gibbons WN, Munkittrick KR, McMaster ME, Taylor WD. 1998. Monitoring aquatic environments receiving industrial effluents using small fish species 2: Comparison between

responses of trout-perch (Percopsis omiscomaycus) and white sucker (Catostomus commersoni) downstream of a pulp mill. Environmental Toxicology and Chemistry 17: 2238-45

Gilbert B, De Souza JP, Fascio M, Kitagawa M, Nascimento SSC, et al. 1970. Schistosomiasis. Protection against infection by terpenoid. Annals of the Brazilian Academy of Science 42: 397-400

Gonny M, Cavaleiro C, Salgueiro L, Casanova J. 2006. Analysis of juniperus communis subsp. alpina needle, berry, wood and root oils by combination of GC, GC/MS and 13C-NMR. Flavour and Fragrance Journal 21: 99-106

Hall A, Bose R, Glavin G, Pinsky C. 1988. Effects of 4-acetylpyridine on stress ulcer formation in mice. Neuroscience Letters 85: 110-12

Heil TP, Lindsay RC. 1990. Environmental and industrial factors relating to flavor tainting of fish in the upper Wisconsin River. Journal of Environmental Science and Health B B25: 527-52

Heinrich M, Barnes J, Gibbons S, Williamson E. 2008. Fundamentals of pharmacognosy and phytotherapy. pp. 308. Churchill Livingstone: London, UK

Hewitt LM, Kovacs TG, Dube MG, MacLatchy DL, Martel PH, et al. 2008. Altered reproduction in fish exposed to pulp and paper mill effluents: roles of individual compounds and mill operating conditions. Environmental Toxicology and Chemistry 27: 682-97

Hewitt LM, Parrott JL, McMaster ME. 2006. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: sources and characteristics of bioactive substances. Journal of Toxicology and Environmental Health Part B: Critical Reviews 9: 341-56

Higuchi T, Ishikawa H, Hasegawa M. 1960. Lignin in cultured tissues of Pinus strobus and Paulownia tomentosa. Mokuzai Gakkaishi 6: 229-32

Hwang JS, Lee SA, Hong SS, Lee KS, Lee MK, et al. 2005. Monoamine oxidase inhibitory components from the roots of Sophora flavescens. Archives of Pharmacal Research 28: 190-4

Ikeda T, Matsumura F, Benjamin DM. 1977. Mechanism of feeding discrimination between matured and juvenile foliage by two species of pine sawflies. Journal of Chemical Ecology 3: 677-94

Iordache AM, Culea M, C Onuc. Characterization of some extracts for therapeutic use by GC/MS. Journal of Physics: Conference Series 182: 012027

Jayne JE. 1953. A study of the alkaline nitrobenzene oxidation of chlorite lignin. Tappi Journal 36: 571-6

Jo YS, Huong DTL, Bae KH, Lee MK, Kim YH. 2002. Monoamine Oxidase Inhibitory Coumarin from Zanthoxylum schinifolium. Planta Medica 68: 84-85

Justo G, Motta M, Martini L. 1975. In vivo effects of acetylcholine on LH secretion. Experientia 31: 598-600

Kah O, Dulka JG, Dubourg P, Thibault J, Peter RE. 1987. Neuroanatomical substrate for the inhibition of gonadotrophin secretion in goldfish: existence of a dopaminergic preoptico-hypophyseal pathway. Neuroendocrinology 45: 451-8

Kalmes M, Neumeyer A, Rio P, Hanenberg H, Fritsche E, et al. 2006. Impact of the arylhydrocarbon receptor on eugenol- and isoeugenol-induced cell cycle arrest in human immortalized keratinocytes (HaCaT). Biological chemistry 387: 1201-7

Kersten PJ, Kopper BJ, Raffa KF, Illman BL. 2006. Rapid analysis of abietanes in conifers. Journal of Chemical Ecology 32: 2679-85

Kim KH, Moon E, Choi SU, Kim SY, Lee KR. 2011. Biological evaluation of phenolic constituents from the trunk of Berberis koreana. Bioorganic & Medicinal Chemistry Letters 21: 2270-73

Kiparissis Y, Hughes R, Metcalfe C, Ternes T. 2001. Identification of the Isoflavonoid Genistein in Bleached Kraft Mill Effluent. Environmetnal Science and Technology 35: 2423-27

Kokubo R, Sakai K, Imamura H. 1990. Secondary metabolites in cell-cultures of woody-plants .2. formation of pinosylvin and monomethyl ether in pinus-thunberII callus and the effect of UV irradiation on their contents. Mokuzai Gakkaishi 36: 1084-8

Kostamo A, Holmbom B, Kukkonen JVK. 2004. Fate of wood extractives in wastewater treatment plants at kraft pulp mills and mechanical pulp mills. Water Research 38: 972-82

Kovacs T, Martel P, Ricci M, Michaud J, Voss R. 2005. Further insights into the potential of pulp and paper mill effluents to affect fish reproduction. Journal of Toxicology and Environmental Health, Part A 68: 1621-41

Kovacs TG, Martel PH, Ricci M. 2007. A shortened adult fathead minnow reproduction test developed for investigation of cause and investigation of solution work involving pulp and paper mill effluents. Water Quality Research Journal of Canada 42: 91-100

Kovacs T, Naish V, O'Connor B, Blaise C, Gagné F, et al. 2010. An ecotoxicological characterization of nanocrystalline cellulose (NCC). Nanotoxicology 4: 255-70

Kovacs TG, Martel PH, O'Connor BI, Parrott JL, McMaster ME, et al. 2011. Kraft mill effluent survey: Progress toward best management practices for reducing effects on fish reproduction. Environmental Toxicology and Chemistry 30: 1421-29

Kovacs TG, Martel PH, O'Connor BI, Hewitt LM, Parrott JL, et al. 2013. A survey of Canadian mechanical pulp and paper mill effluents: Insights concerning the potential to affect fish reproduction. Journal of Environmental Science and Health, Part A 48: 1178-89

Krauze-Baranowska M, Mardarowicz M, Wiwart M, Poblocka L, Dynowska M. 2002. Antifungal activity of the essential oils from some species of the genus Pinu. Zeitschrift fur Naturforschung C 57: 478-482

Krishnaswamy S, Hao Q, von Moltke LL, Greenblatt DJ, Court MH. 2004. Evaluation of 5-hydroxytryptophol and other endogenous serotonin (5-hydroxytryptamine) analogs as substrates for UDP-glucuronosyltransferase 1A6. Drug Metabolism and Disposition 32: 862-69

Krsmanovic LZ, Mores N, Navarro CE, Saeed SA, Arora KK, et al. 1998. Muscarinic regulation of intracellular signaling and neurosecretion in gonadotropin-releasing hormone neurons. Endocrinology 139: 4037-43

Kulkarni S, Bhutani M, Bishnoi M. 2008. Antidepressant activity of curcumin: involvement of serotonin and dopamine system. Psychopharmacology 201: 435-42

LaFlamme R. 1979. Polycyclic aromatic hydrocarbons in the sedimentary environment. MSc Thesis: Tufts University

Lakhan S, Vieira K. 2010. Nutritional and herbal supplements for anxiety and anxiety-related disorders: systematic review. Nutrition Journal 9: 42

Langmead CJ, Watson J, Reavill C. 2008. Muscarinic acetylcholine receptors as CNS drug targets. Pharmacology & Therapeutics 117: 232-43

Lapidus KA, Soleimani L, Murrough JW. 2013. Novel glutamatergic drugs for the treatment of mood disorders. Neuropsychiatric Disease and Treatment 9: 1101-12

Leduc C, Coonishish J, Haddad P, Cuerrier A. 2006. Plants used by the Cree Nations of Eeyou Istchee (Quebec, Canada) for the treatment of diabetes: A novel approach in quantitative ethnobotany. Journal of Ethnopharmacology 105: 55 - 63

Lee MK, Hwang BY, Lee SA, Oh GJ, Choi WH, et al. 2003. 1-Methyl-2-undecyl-4(1H)-quinolone as an irreversible and selective inhibitor of type B monoamine oxidase. Chemical and Pharmaceutical Bulletin 51: 409-11

León-Olea M, Martyniuk CJ, Orlando EF, Ottinger MA, Rosenfeld CS, et al. 2014. Current concepts in neuroendocrine disruption. General and Comparative Endocrinology 203: 158-73

Leppänen H, Marttinen S, Oikari A. 1998. The use of fish bile metabolite analyses as exposure biomarkers to pulp and paper mill effluents. Chemosphere 36: 2621-34

Leusch FDL, MacLatchy DL. 2003. In vivo implants of [beta]-sitosterol cause reductions of reactive cholesterol pools in mitochondria isolated from gonads of male goldfish (Carassius auratus). General and Comparative Endocrinology 134: 255-63

Li F, Tsien JZ. 2009. Memory and the NMDA Receptors. New England Journal of Medicine 361: 302-03

Lindberg LE, Willför SM, Holmbom BR. 2004. Antibacterial effects of knotwood extractives on paper mill bacteria. Journal of Industrial Microbiology Biotechnology 31: 137-47

Lindstedt G. 1949. Constituents of pine heartwood. XIV. Heartwood of Pinus monticola Dougl. Acta Chemica Scandinavica 3: 1147-52

Lindstedt G, Misiorny A. 1951. Constituents of pine heartwood. XXV. Investigation of 48 Pinus species by paper-partition chromatography. Acta Chemica Scandinavica 5: 121-8.

Linton ED, Scuton DA, McKinley RS. 2005. Physiological effects of thermomechanical newsprint mill effluent on Atlantic salmon (Salmo salar L.). Ecotoxicology and Environmental Safety 62: 317-30

Liu AC, Tran HG, Zhang EE, Priest AA, Welsh DK, et al. 2008. Redundant function of REV-ERBα and β and non-essential role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms. PLoS Genetics 4: e1000023

Loziene K, Labokas J, Venskutonis PR, Mazdzieriene R. 2010. Chromatographic evaluation of the composition of essential oil and α-pinene enantiomers in Juniperus communis L. berries during ripening. Journal of Essential Oil Research 22: 453-458

Makris SP, Banerjee S. 2002. Fate of resin acids in pulp mill secondary treatment systems. Water Research 36: 2878-82

Manville JF. 1975. Juvabione and its analogs. Juvabione and Δ4'-dehydrojuvabione isolated from the whole wood of Abies balsamea, have the R,R stereoconfigurations, not the R,S. Canadian Journal of Chemistry 53: 1579-85

Martel PH, Kovacs TG, O'Connor BI, Semeniuk S, Hewitt LM, et al. 2011. Effluent monitoring at a bleached kraft mill: Directions for best management practices for eliminating

effects on fish reproduction. Journal of Environmental Science and Health, Part A 46: 833-43

Martin AM, Queiroz EF, Marston A, Hostettmann K. 2006. Labdane diterpenes from Juniperus communis L. berries. Phytochemical Analysis 17: 32-35

McMaster ME, Van Der Kraak GJ, Munkittrick KR. 1996. An Epidemiological Evaluation of the Biochemical Basis for Steroid Hormonal Depressions in Fish Exposed to Industrial Wastes. Journal of Great Lakes Research 22: 153-71

McMaster ME, Hewitt LM, Parrott JL. 2006. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: Field studies and mechanistic research. Journal of Toxicology and Environmental Health Part B: Critical Reviews 9: 319-39

Mellanen P, Petänen T, Lehtimäki J, Mäkelä S, Bylund G, et al. 1996. Wood-derived estrogens: Studies in vitro with breast cancer cell lines and in vivo in trout. Toxicology and Applied Pharmacology 136: 381-88

Meriläinen P, Oikari A. 2008. Exposure assessment of fishes to a modern pulp and paper mill effluents after a black liquor spill. Environmental Monitoring and Assessment 144: 419-35

Meyers PA, Leenheer MJ, Bourbonniere RA. 1995. Diagenesis of vascular plant organic matter components during burial in lake sediments. Aquatic Geochemistry 1: 35-52

Michl G, Rettenbacher C, Haslinger E. 1988. Synthesis of 12-methoxyabietic acid methyl ester, a feeding deterrent of the larch sawfly Pristiphora erichsonii (Hartig). Monatshefte fur Chemie 119: 833-8

Milestone CB, Orrego R, Scott PD, Waye A, Kohli J, et al. 2012. Evaluating the potential of effluents and wood feedstocks from pulp and paper mills in Brazil, Canada, and New Zealand to affect fish reproduction: Chemical profiling and in vitro assessments. Environmental Science & Technology 46: 1849-58

Mullally M, Kramp K, Cayer C, Saleem A, Ahmed F, et al. 2011. Anxiolytic activity of a supercritical carbon dioxide extract of Souroubea sympetala (Marcgraviaceae). Phytotherapy Research 25: 264-70

Munkittrick KR, McMaster ME, McCarthy LH, Servos MR, Van Der Kraak GJ. 1998. An overview of recent studies on the potential of pulp-mill effluents to alter reproductive parameters in fish. Journal of Toxicology and Environmental Health Part B: Critical Reviews 1: 347-71

Naoi M, Nagatsu T. 1987. Quinoline and quninaldine as naturally occurring inhibitors specific for type A monoamine oxidase. Life Sciences 40: 1075-82

Nicholson RA, Lees G, Zheng J, Verdon B. 1999. Inhibition of GABA-gated chloride channels by 12,14-dichlorodehydroabietic acid in mammalian brain. British Journal of Pharmacology 126: 1123-32

Niemann GJ. 1972. Phenolics from Larix needles. IV. Constituents of Larix laricina. Acta Botanica Neerlandica 21: 549-54

Nierop KGJ, Preston CM, Kaal J. 2005. Thermally assisted hydrolysis and methylation of purified tannins from plants. Analytical Chemistry 2005 77: 5604-14

NRCan. 2013. Canadian Forestry Service. http://cfs.nrcan.gc.ca/pages/52. Natural Resources Canada

O'Connor BI, Kovacs TG, Voss RH. 1992. The effect of wood species composition on the toxicity of simulated mechanical pulping effluents. Environmental Toxicology and Chemistry 11: 1259-70

Ohigashi H, Wagner MR, Matsumura F, Benjamin DM. 1981. Chemical basis of differential feeding behavior of the larch sawfly, Pristiphora erichsonii (Hartig). Journal of Chemical Ecology 7: 599-614

Oikari A, Holmbom B, Bister H. 1982. Uptake of resin acids into tissues of trout (Salmo gairdneri Richardson). Annales Zoologici Fennici 19: 61-64

Oikari A, Lönn B-E, Castrén M, Nakari T, Snickars-Nikinmaa B, et al. 1983. Toxicological effects of dehydroabietic acid (DHAA) on the trout, Salmo gairdneri Richardson, in fresh water. Water Research 17: 81-89

Olsvik PA, Lie KK, Hevroy EM. 2007. Do anesthetics and sampling strategies affect transcription analysis of fish tissues? BMC Molecular Biology 8: 48

Omar S, Lemonnier B, Jones N, Ficker C, Smith ML, et al. 2000. Antimicrobial activity of extracts of eastern North American hardwood trees and relation to traditional medicine. Journal of Ethnopharmacology 73: 161-70

Oros DR, Simoneit BRT. 2001. Identification and emission factors of molecular tracers in organic aerosols from biomass burning. Part 1. Temperate climate conifers. Applied Geochemistry 16: 1513-44

Pakdel H, Couture G, Roy C. 1994. Vacuum pyrolysis of bark residues and primary sludges. Tappi Journal 77: 205-11.

Park EJ, Min HY, Chung HJ, Ahn YH, Pyee JH, et al. 2011. Pinosylvin suppresses LPS-stimulated inducible nitric oxide synthase expression via the MyD88-independent, but TRIF-

dependent downregulation of IRF-3 signaling pathway in mouse macrophage cells. Cellular Physiology and Biochemistry 27: 353-62

Parrott J, Kohli J, Sherry J, Hewitt L. 2011. In vivo and in vitro mixed-function oxygenase activity and vitellogenin induction in fish and in fish and rat liver cells by stilbenes isolated from Scotch pine (Pinus sylvestris). Archives of Environmental Contamination and Toxicology 60: 116-23

Parrott JL. 2005. Overview of methodology and endpoints in fathead minnow lifecycle tests assessing pulp and paper mill effluents. Water Quality Research Journal of Canada 40: 334-46

Parrott JL, McMaster ME, Hewitt LM. 2006. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: Development and application of fish bioassays. Journal of Toxicology and Environmental Health Part B: Critical Reviews 9: 297-317

Pasay C, Mounsey K, Stevenson G, Davis R, Arlian L, et al. 2010. Acaricidal activity of eugenol based compounds against scabies mites. PLoS ONE 5: e12079

Pelissero C, Bennetau B, Babin P, Le Menn F, Dunogues J. 1991. The estrogenic activity of certain phytoestrogens in the siberian sturgeon Acipenser baeri. The Journal of Steroid Biochemistry and Molecular Biology 38: 293-99

Pepper JM, Supathna P. 1978. Lignin and related compounds. VI. A study of variables affecting the hydrogenolysis of sprucewood lignin using a rhodium-on-charcoal catalyst. Canadian Journal of Chemistry 56: 899-902

Perng WR. 1986 Effects of vanillin-formaldehyde resin treatments on dimensional stability of wood. Mokuzai Gakkaishi 32: 439-47

Peter RE. 1982. Neuroendocrine Control of Reproduction in Teleosts. Canadian Journal of Fisheries and Aquatic Sciences 39: 48-55

Peter RE, Chang JP, Nahorniak CS, Omeljaniuk RJ, Sokolowska M, et al. 1986. Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Progress in Hormone Research. 42: 513-48

Peter RE, Yu KL. 1997. Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. Reviews in Fish Biology and Fisheries 7: 173-97

Phelan M, Aherne SA, Wong A, O'Brien NM. 2009. Bioactive properties of wood knot extracts on cultured human cells. Journal of Medicinal Food 12: 1245-51.

Pietarinen SP, Willfor SM, Ahotupa MO, Hemming JE, Holmbom BR. 2006 Knotwood and bark extracts: strong antioxidants from waste materials. Journal of Wood Science 52: 436-44

Pinsky C, Bose R. 1987. Longlasting anticonvulsant effects following single injection of acetylpyridine congeners in mice. Proceedings of the Canadian Federation of Biological Societies 30: 111

Polcin J, Rapson WH. 1971. Sapwood and heartwood groundwood of western hemlock and jack pine. Part II. Heat stability of extractives. Pulp and Paper Magazine of Canada 72: T324-30

Poncsak S, Kocaefe D, Simard F, Pichette A. 2009. Evolution of extractive composition during thermal treatment of Jack Pine. Journal of Wood Chemistry and Technology 29: 251-64

Popesku JT, Martyniuk CJ, Mennigen J, Xiong H, Zhang D, et al. 2008. The goldfish (Carassius auratus) as a model for neuroendocrine signaling. Molecular and Cellular Endocrinology 293: 43-56

Popesku JT, Tan EYZ, Martel PH, Kovacs TG, Rowan-Carroll A, et al. 2010. Gene expression profiling of the fathead minnow (Pimephales promelas) neuroendocrine brain in response to pulp and paper mill effluents. Aquatic Toxicology 99: 379-88

Powell JS, Raffa KF. 1999. Effects of selected Larix laricina terpenoids on Lymantria dispar (Lepidoptera: Lymantriidae) development and behavior. Environmental Entomology 28: 148-54

Raiber S, Schröder G, Schröder J. 1995. Molecular and enzymatic characterization of two stilbene synthases from Eastern white pine (Pinus strobus) A single Arg/His difference determines the activity and the pH dependence of the enzymes. FEBS Letters 361: 299-302

Rajakumar DV, Rao MNA. 1993. Dehydrozingerone and isoeugenol as inhibitors of lipid peroxidation and as free radical scavengers. Biochemical Pharmacology 46: 2067-72

San Feliciano A, Caballero E, Del Rey B, Sancho I. 1991. Diterpene acids from Juniperus communis subsp. Hemisphaerica. Phytochemistry 30: 3134-6

Santamour FS Jr. 1967. Notes on pine resins and pest resistance. Morris Arboretum Bulletin 18: 82-6

Schoenhofen IC, McNally DJ, Brisson J-R, Logan SM. 2006. Elucidation of the CMP-pseudaminic acid pathway in Helicobacter pylori: synthesis from UDP-N-acetylglucosamine by a single enzymatic reaction. Glycobiology 16: 8C-14C

Scholey A, Ossoukhova A, Owen L, Ibarra A, Pipingas A, et al. 2010. Effects of American ginseng (Panax quinquefolius) on neurocognitive function: an acute, randomised, double-blind, placebo-controlled, crossover study. Psychopharmacology 212: 345-56

Schuh BA, Benjamin DM. 1984. The chemical feeding ecology of Neodiprion dubiosus Schedl, N. rugifrons Midd., and N. lecontei (Fitch) on jack pine (Pinus banksiana Lamb.). Journal of Chemical Ecology 10: 1071-9

Scoggan H. 1978. The flora of Canada - Part 2, Pteridophyta. Gymnospermae. Monocotyledoneae. National Museums of Canada: Ottawa, Can.

Sherif FM, Saleem Ahmed S. 1995. Basic aspects of GABA-transaminase in neuropsychiatric disorders. Clinical Biochemistry 28: 145-54

Sinclair GD, Dymond DK. 1973. Distribution and composition of extractives in jack pine trees. Canadian Journal of Forest Research 3: 516-21

Sloley BD, Kah O, Trudeau VL, Dulka JG, Peter RE. 1992. Amino acid neurotransmitters and dopamine in brain and pituitary of the goldfish: Involvement in the regulation of gonadotropin secretion. Journal of Neurochemistry 58: 2254-62

Slominski A, Tobin DJ, Zmijewski MA, Wortsman J, Paus R. 2008. Melatonin in the skin: synthesis, metabolism and functions. Trends in Endocrinology & Metabolism 19: 17-24

Smith GB, Olsen RW. 1995. Functional domains of GABAA receptors. Trends in Pharmacological Sciences 16: 162-68

Stacey N, Sorensen P. 2005. Reproductive Pheromones In Fish Physiology, ed. RWW Katherine A. Sloman, B Sigal, pp. 359-412. Academic Press: San Diego, USA

Suga T, Ohta S, Munesada K, Kurokawa M, Shimizu M, et al. 1993. Endogenous pine wood nematicidal substances in pines, Pinus-massoniana, P. strobus and P. palustris. Phytochemistry 33: 1395-401

Sulea D, Leca M. 2009. Collagen-thuja tincture biomaterials for wound treatment 1. Analysis of the volatile fraction from Thuja columnaris tincture by GC-MS. Revue Roumaine de Chimie 54; 1097-101

Suzuki K, Nagahama Y, Kawauchi H. 1988. Steroidogenic activities of two distinct salmon gonadotropins. General and Comparative Endocrinology 71: 452-58

Swanson P, Suzuki K, Kawauchi H, Dickhoff WW. 1991. Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. Biology of Reproduction 44: 29-38

Tabassum I, Rizvi S, Siddiqui Z. 2010. Effects of Ocimum sanctum and Camellia sinensis on stress-induced anxiety and depression in male albino Rattus norvegicus. Indian Journal of Pharmacology 42: 283-88

Tatsuzaki J, Bastow KF, Nakagawa-Goto K, Nakamura S, Itokawa H, et al. 2006. Dehydrozingerone, chalcone, and isoeugenol analogues as in vitro anticancer agents. Journal of Natural Products 69: 1445-49

Tezuka Y, Morikawa K, Li F, Auw L, Awale S, et al. 2010. Cytochrome P450 3A4 inhibitory constituents of the wood of Taxus yunnanensis. Journal of Natural Products 74: 102-05

Tomlin ES, Antonejevic E, Alfaro RI, Borden JH. 2000. Changes in volatile terpene and diterpene resin acid composition of resistant and susceptible white spruce leaders exposed to simulated white pine weevil damage. Tree Physiology 20: 1087-95

Tremblay L, Van Der Kraak G. 1999. Comparison between the effects of the phytosterol β-sitosterol and pulp and paper effluents on sexually immature rainbow trout. Environmental Toxicology and Chemistry 18: 329-36

Trudeau V. 1997. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, Carassius auratus. Reviews of Reproduction 2: 55-68

Trudeau VL, Spanswick D, Fraser EJ, Larivière K, Crump D, et al. 2000. The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. Biochemistry and Cell Biology 78: 241-59

Uprety Y, Asselin H, Dhakal A, Julien N. 2012. Traditional use of medicinal plants in the boreal forest of Canada: review and perspectives. Journal of Ethnobiology and Ethnomedicine 8: 7

Van den Heuvel MR, Martel PH, Kovacs TG, MacLatchy DL, Van Der Kraak G, et al. 2010. Evaluation of short-term fish reproductive bioassays for predicting effects of a Canadian bleached kraft mill effluent. Water Quality Research Journal of Canada 45: 11

Van Der Kraak GJ, Munkittrick KR, McMaster ME, Portt CB, Chang JP. 1992. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. Toxicology and Applied Pharmacology 115: 224-33

Vichi S, Riu-Aumatell M, Mora-Pons M, Guadayol JM, Buxaderas S, et al. 2007. HS-SPME coupled to GC/MS for quality control of Juniperus communis L. berries used for gin aromatization. Food Chemistry 105: 1748-54

Vichi S, Aumatell MR, Buxaderas S, López-Tamames E. 2008. Assessment of some diterpenoids in commercial distilled gin. Analytica Chimica Acta 628: 222-9

Villeneuve DL, Blake LS, Brodin JD, Cavallin JE, Durhan EJ, et al. 2008. Effects of a 3β-hydroxysteroid dehydrogenase inhibitor, trilostane, on the fathead minnow reproductive axis. Toxicological Sciences 104: 113-23

Von Rudloff E, Sato A.1963. The heartwood extractives of Pinus banksiana. Canadian Journal of Chemistry 41: 2165-74

Von Rudloff E. 1987. The volatile twig and leaf oil terpene compositions of three western North American larches, Larix laricina, Larix occidentalis, and Larix lyallii. Journal of Natural Products 50: 317-21

Voss RH, Rapsomatiotis A. 1985. An improved solvent-extraction based procedure for the gas chromatographic analysis of resin and fatty acids in pulp mill effluents. Journal of Chromatography A 346: 205-14

Wagner MR, Benjamin DM, Clancy KM, Schuh BA. 1983. Influence of diterpene resin acids on feeding and growth of larch sawfly, Pristiphora erichsonii (Hartig). Journal of Chemical Ecology 9: 119-27

Wartman CA, Hogan NS, Hewitt LM, McMaster ME, Landman MJ, et al. 2009. Androgenic effects of a Canadian bleached Kraft pulp and paper effluent as assessed using threespine stickleback (Gasterosteus aculeatus). Aquatic Toxicology 92: 131-39

Waye A, Trudeau VL. 2011. Neuroendocrine Disruption: More Than Hormones are Upset. Journal of Toxicology and Environmental Health, Part B 14: 270-91

Waye A, Annal M, Tang A, Picard G, Harnois F, et al. 2014a. Canadian boreal pulp and paper feedstocks contain neuroactive substances that interact in vitro with GABA and dopaminergic systems in the brain. Science of The Total Environment 468–469: 315-25

Waye A, Lado WE, Martel PH, Arnason JT, Trudeau VL. 2014b. Ovulation but not milt production is inhibited in fathead minnows (Pimephales promelas) exposed to a reproductively inhibitory pulp mill effluent. Reproductive Biology and Endocrinology 12: 43

Weintraub D, Burn DJ. 2011. Parkinson's disease: The quintessential neuropsychiatric disorder. Movement Disorders 26: 1022-31

Werner J, Ouellet JD, Cheng CS, Ju Y-J, Law RD. 2010. Pulp and paper mill effluents induce distinct gene expression changes linked to androgenic and estrogenic responses in the fathead minnow (Pimephales promelas). Environmental Toxicology and Chemistry 29: 430-39

Willfoer SM, Ahotupa MO, Hemming JE, Reunanen MHT, Eklund PC, et al. 2003. Antioxidant activity of knotwood extractives and phenolic compounds of selected tree species. Journal of Agricultural Food Chemistry 51: 7600-6

Yaron Z. 1995. Endocrine control of gametogenesis and spawning induction in the carp. Aquaculture 129: 49-73

Youdim MBH. 2010. Why do we need multifunctional neuroprotective and neurorestorative drugs for Parkinson's and Alzheimer's diseases as disease modifying agents. Experimental Neurobiology 19: 1-14

Zheng J, Nicholson RA. 1996. Influence of two naturally occurring abietane monocarboxylic acids (resin acids) and a chlorinated derivative on release of the inhibitory neurotransmitter gamma-aminobutyric acid from trout brain synaptosomes. Bulletin of Environmental Contamination and Toxicology 56: 114-20

Zheng J, Nicholson RA. 1998. Action of resin acids in nerve ending fractions isolated from fish central nervous system. Environmental Toxicology and Chemistry 17: 1852-59

Zinkel DF, Magee TV. 1987. Diterpene resin acids from the needle oleoresin of Pinus strobus. Phytochemistry 26: 769-74

Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O. 2010. Neuroendocrinology of reproduction in teleost fish. General and Comparative Endocrinology 165: 438-55

Appendix 1

Journal of Toxicology and Environmental Health, Part B, 14:270–291, 2011

ISSN: 1093-7404 print / 1521-6950 online
DOI: 10.1080/10937404.2011.578273

Taylor & Francis
Taylor & Francis Group

NEUROENDOCRINE DISRUPTION: MORE THAN HORMONES ARE UPSET

Andrew Waye, Vance L. Trudeau

Centre for Advanced Research in Environmental Genomics, Department of Biology, University of Ottawa, Ottawa, Ontario, Canada

Only a small proportion of the published research on endocrine-disrupting chemicals (EDC) directly examined effects on neuroendocrine processes. There is an expanding body of evidence that anthropogenic chemicals exert effects on neuroendocrine systems and that these changes might impact peripheral organ systems and physiological processes. Neuroendocrine disruption extends the concept of endocrine disruption to include the full breadth of integrative physiology (i.e., more than hormones are upset). Pollutants may also disrupt numerous other neurochemical pathways to affect an animal's capacity to reproduce, develop and grow, or deal with stress and other challenges. Several examples are presented in this review, from both vertebrates and invertebrates, illustrating that diverse environmental pollutants including pharmaceuticals, organochlorine pesticides, and industrial contaminants have the potential to disrupt neuroendocrine control mechanisms. While most investigations on EDC are carried out with vertebrate models, an attempt is also made to highlight the importance of research on invertebrate neuroendocrine disruption. The neurophysiology of many invertebrates is well described and many of their neurotransmitters are similar or identical to those in vertebrates; therefore, lessons learned from one group of organisms may help us understand potential adverse effects in others. This review argues for the adoption of systems biology and integrative physiology to address the effects of EDC. Effects of pulp and paper mill effluents on fish reproduction are a good example of where relatively narrow hypothesis testing strategies (e.g., whether or not pollutants are sex steroid mimics) have only partially solved a major problem in environmental biology. It is clear that a global, integrative physiological approach, including improved understanding of neuroendocrine control mechanisms, is warranted to fully understand the impacts of pulp and paper mill effluents. Neuroendocrine disruptors are defined as pollutants in the environment that are capable of acting as agonists/antagonists or modulators of the synthesis and/or metabolism of neuropeptides, neurotransmitters, or neurohormones, which subsequently alter diverse physiological, behavioral, or hormonal processes to affect an animal's capacity to reproduce, develop and grow, or deal with stress and other challenges. By adopting a definition of neuroendocrine disruption that encompasses both direct physiological targets and their indirect downstream effects, from the level of the individual to the ecosystem, a more comprehensive picture of the consequences of environmentally relevant EDC exposure may emerge.

Following the first WWF Wingspread Conference in 1991 and the publication of Theo Colborn's book *Our Stolen Future* in 1996, there has been increasing public concern about how natural or synthetic compounds interact with the hormonal systems of humans and wildlife. In the last two decades the scientific community has continued to explore the presence and effects of endocrine-disrupting chemicals (EDC) in the environment (Colborn et al. 1993; Vos et al. 2000; Porte et al. 2006; Hotchkiss et al. 2008).

Address correspondence to Dr. Vance L. Trudeau, Centre for Advanced Research in Environmental Genomics, Department of Biology, University of Ottawa, Ottawa, Ontario, K1N 6N5, Canada. E-mail: trudeauv@uottawa.ca

The U.S. Environmental Protection Agency (EPA) defines endocrine disruptors as chemicals that either mimic or block the effects of hormones at the target receptor/tissue or by directly stimulating or inhibiting production of hormones by the endocrine system (U.S. EPA 2007). It is our intention to define "neuroendocrine disruption" for the broader community interested in endocrine disruption and ecotoxicology in order to describe how environmental pollutants may impact brain functions as they relate to hormonal systems. To our knowledge it is the first such attempt, and will no doubt require extensive debate and refinement in the coming years. Indeed, the purpose of the first symposium on Neuroendocrine Effects of Endocrine Disruptors (NEED) is to present existing data and begin the debate on the emerging concept of neuroendocrine disruption. We realize that this term may be too general for some but perfect for others. It succinctly encompasses our view of how pollutants disrupt development and physiological functions in animals.

The field of neuroendocrinology has expanded considerably since the first dedicated meetings in the early 1970s. One definition consisting of elements from various mission statements of journals and societies could serve well in this discussion of neuroendocrine disruption. Neuroendocrinology is the study of the interplay between the endocrine and nervous systems that control all bodily processes in vertebrates and invertebrates, and its expanding interface with the regulation of behavioral, cognitive, developmental, immunological, degenerative, and metabolic processes. Therefore, neuroendocrine disruption from an environmental perspective comprises all these elements and how they are affected by biologically active pollutants of diverse origins.

There is an expanding body of evidence that industrial, agricultural, and pharmaceutical chemicals exert effects on vertebrate and invertebrate neuroendocrine systems (Tables 1–4). One part of a definition might be that neuroendocrine disruptors exert their effects as agonists/antagonists of neuropeptides, neurotransmitters, or neurohormones, thereby affecting hormonal systems. There is also evidence that some environmental pollutants disrupt the synthesis or metabolism of neurotransmitters that regulate hormone release. These changes result in an altered neurophysiological state, which subsequently influences many downstream systems under control of the neuroendocrine brain. Neuroendocrine systems integrate internal (e.g., hormones, metabolic signals) and external (e.g., pheromones, temperature, photoperiod) stimuli to allow physiological and behavioral adaptation to the environment. Therefore, neuroendocrine disruption extends the concept of endocrine disruption to include the full breadth of integrative physiology—that is, neuroendocrine disruption is more than just hormones. It is possible that pollutants disrupt numerous other neurochemical pathways, upsetting diverse physiological and behavioral processes to affect an animal's capacity to reproduce, grow, or deal with stress and other challenges.

Much in the way that endocrine disruption is different from classical toxicology, neuroendocrine disruption is distinguishable from neurotoxicology. Neurotoxicologists study chemical insults and mechanisms underlying subsequent neuronal cell death, which eventually lead to the failure of key regulatory systems and death of exposed individuals. Rather, the consequences of disrupting the complex neurohormonal brain–pituitary–target organ communication systems are within the domain of neuroendocrine disruption.

It is difficult to pinpoint the first use of the phrase "neuroendocrine disruption." However, studies of pollutants on the brain–pituitary complex most certainly predate 1991 (Singh and Singh 1980; Smith 1983), when the term "endocrine disruption" was introduced (Gore 2010). It appears that serious consideration of the hypothalamus as a main EDC target was likely developing in the mid to late 1990s, since some of the first papers specifically addressing this issue in fish, frogs, turtles, and mammals

TABLE 1. In Vivo Neuroendocrine Disruption Observed in Vertebrates by Pharmaceuticals and Personal Care Products Released in Municipal Effluents and Detected in the Environment

Disruptor	Species	Effect	Treatment
Ethynylestradiol	*Xenopus tropicalis*	Decreased ER-α expression in brain, skewed female sex ratio with females lacking oviduct	1–100 n*M* waterborne (Pettersson et al. 2006)
	Danio rerio	Differential expression changes in liver and tel measured by microarray	10 ng/L waterborne, males (Martiniuk et al. 2007)
	Carassius auratus	Increased CYP19B expression in hyp and tel	10 ng/L waterborne, males (Martiniuk et al. 2006)
	Oryzias latipes	Decreased GnRH-R in brain	5000 ng/L waterborne, males (Zhang et al. 2008a)
		Decreased AR-α in brain, impaired sexual behavior	5 ng/L waterborne, males (Zhang et al. 2008a)
	Oreochromis niloticus	Decreased IGF-1 expression in female brain	125 μg/g b.w. dietary (Shved et al. 2007)
		Normal IGF-1 expression patterns disrupted in males and females, normal ER-α expression patterns disrupted in males and suppressed in females	5 and 25 ng/L waterborne (Shved et al. 2008)
Fadrozole	*C. auratus*	Differential gene expression in hyp and tel measured by microarray. Downregulation of CYP19B in hyp and tel	50 μg/L waterborne, females (Zhang et al. 2009)
Clotrimazole	*X. tropicalis*	Decreased CYP19 activity in brain of developing tadpoles	375 n*M* waterborne (Gyllenhammar et al. 2009)
Ketoconazole	*O. latipes*	Decreased GnRH-R expression in brain	3–300 μg/L females (Zhang et al. 2008b)
SSRIs	*vertebrates*	Neuroendocrine disruption	(Mennigen et al. 2011)

Note. Hyp, hypothalamus, Tel, telencephalon, GnRH-R, GnRH receptor, IGF-1, insulin-like growth factor 1, ER-α, estrogen receptor alpha, AR-α, androgen receptor alpha, CYP19B, aromatase B, and SSRI, selective serotonin reuptake inhibitor.

were being published around that time and early in the new millenium (Van Der Kraak et al. 1992; Cooper et al. 1999; Khan and Thomas 2001; Trudeau et al. 2002; Crump et al. 2002). One could find the term "neuroendocrine disruption" in the titles of five papers. Two were on molluscs (Gagne et al. 2007a; 2007b), one on the antidepressant mianserin that was phased out of use in most markets (van der Ven et al. 2006), a review on sexual maturation (Bourguignon et al. 2010), and an editorial by Gore and Patisaul (2010). In none of these papers was "neuroendocrine disruption" conceptualized or a definition proposed. These publications are recent, which highlights the novelty and importance of this emerging issue. Around the same time as the first of these publications, a definition was posted on our website (www.teamendo.ca/Community/Our+Lab+Members/1064.aspx), and Gore (2008) and Zoeller (2008) began debating and discussing neuroendocrine targets of EDC in 2008. Regardless, the historical foundations leading any discussion of neuroendocrine effects of EDC are elegantly presented by Gore and Patisaul (2010). In the same issue of *Frontiers in Neuroendocrinology* there are articles covering the effects of EDC on reproductive health, neuroendocrine function, energy balance, and other topics in mammalian models and humans. Here a broader view is taken, and disparate data are examined from numerous invertebrate and vertebrate model systems.

Human activities have introduced neuroendocrine disruptors to the air, water, and soil globally through extensive use of pharmaceuticals and pesticides and through industrial activities that create and/or emit neuroactive byproducts. This is not an exhaustive review, but several examples are used to illustrate key points to provide the framework for future debate on the concept of neuroendocrine disruption.

TABLE 2. In Vivo Neuroendocrine Disruption Observed in Vertebrates by Chlorinated Pesticides

Disruptor	Species	Effect	Treatment
Atrazine	*Rattus norvegicus*	Loss of brain-stimulated release of prolactin, inhibition of LH and FSH	200 mg/kg/d dietary, females (Goldman et al. 1999)
	Coturnix japonica	Increased GnRH, nonsignificant positive trend with increased exposure	0.5, 5, 50 μg injections to eggs (Ottinger et al. 2009)
Methoxychlor	*R. norvegicus*	Disrupted prolactin secretory patterns via inhibition of DA in hyp, decreased circulating LH	25 mg/kg/d dietary, females (Lafuente et al. 2000)
		Increased NA and disrupted 5-HT levels in different parts of hyp	25 mg/kg/d dietary, females (Lafuente et al. 2008)
	C. japonica	Increased GnRH in 5 ppm treated males, 0.5 ppm treated females	0.5 and 5 ppm dietary (Ottinger et al. 2005)
		Reduced NA and adrenalin in affected individuals	0.5 and 5 ppm dietary, females (Ottinger et al. 2009)
	Mus domesticus	Female offspring displayed typical male exploratory behavior, decreased D1-like receptor density in nucleus accumbens and olfactory tubercle.	20 μg/kg/d dietary to mothers (Panzica et al. 2007
Vinclozolin	*Oryctolagus cuniculus*	Fewer GnRH neurons and increased calbindin neurons in reproductive brain centres of offspring	10 mg/kg/d dietary to mothers (Bisenius et al. 2006)
		Decreased FSH and impaired sexual behavior and loss of pituitary GnRH sensitivity in male pups	7.2 and 72 mg/kg/d dietary to mothers (Veeramachaneni et al. 2006)
	C. japonica	Disruption to GnRH levels	25, 50, amd100 ppm injection to eggs (McGary et al. 2001)
Prochloraz	*Oryzias latipes*	Decreased GnRH, GnRH-R, and CYP19 in brain, reduced fecundity	3–300 μg/L waterborne to females (Zhang et al. 2008b)
Dieldrin	*Micropterus salmoides*	Transcriptomic and proteomic changes in hyp	10 mg/kg injections (Martyniuk et al. 2010a)
		Decreased ER-β expression in hyp	2.95 ppm dietary (Martyniuk et al. 2010b)

Note. GnRH, gonadotropin-releasing hormone, Hyp, hypothalamus, Tel, telencephalon, GnRH-R, GnRH receptor, DA, dopamine, 5-HT, serotonin, LH, luteinizing hormone, FSH, follicle-stimulating hormone, NA, noradrenaline, and ER-β, estrogen receptor beta.

NEUROENDOCRINE CONTROL AND ITS DISRUPTION BY ENVIRONMENTAL CONTAMINANTS IN VERTEBRATES

Many of the peripheral endocrine glands including thyroid, adrenal, and gonads are directly under the control of the pituitary gland. Chemical messengers such as releasing hormones and neurotransmitters from the hypothalamic regions in the brain send signals to secretory cells within specific regions of the anterior and intermediate pituitary to stimulate the release of numerous trophic hormones such as thyrotropic hormone, adrenocorticotropic hormone, gonadotropins, prolactin, or growth hormone. Many of these chemical messengers such as the neurotransmitter serotonin or the catecholamines play a role not only as chemical messengers in the brain, but as hormones themselves in the peripheral tissues. The neurohypophyseal neuropeptides oxytocin and vasopressin and their homologues are produced in the hypothalamus and released by nerve terminals situated in the posterior pituitary. These secretions from the pituitary control hormone release from endocrine glands, and these hormones then exert their influence on target tissues to elicit specific effects. Inputs at each level of endocrine control in this system may be endogenous as in the case of homeostasis via feedback mechanisms or exogenous in the case of perceptible changes in environmental factors, such as photoperiod, temperature, or population stresses. Signals from pollutants also affect an organism at all four levels of the endocrine system from brain, pituitary, endocrine gland, and/or target tissue to result in behavioral and physiological

TABLE 3. In Vivo Neuroendocrine Disruption Observed in Vertebrates by Industrial Contaminants

Disruptor	Species	Effect	Treatment
Cadmium	*R. norvegicus*	Disruption of DA, 5-HT, and NA in different brain regions, and disruption of FSH, LH, ACTH, prolactin, and TSH, depending on route, dose, and time of exposure	(Lafuente et al. 2000a; Lafuente et al. 2000b; Lafuente et al. 2001a; Lafuente et al. 2001b; Lafuente et al. 2003)
	M. salmoides	Increased GnRH in whole brain	67 ng/kg Injection, males (Martiniuk et al. 2009)
Cadmium and lead	*R. norvegicus*	Decreased DA and 5-HT from cadmium recovered when treated with both lead and cadmium at the same time. NA decreased by lead, cadmium, and lead and cadmium. Decreased LH and FSH from cadmium and cadmium and lead exposures.	0.05 mg/kg injection, females (Pillai et al. 2003)
Methylmercury	*Neovison vison*	Disruption of GABA levels	
	fish	Neuroendocrine control of reproduction, neurotransmitter systems	0.1–2 ppm dietary, males (Basu et al. 2010) (Castoldi et al. 2001; Johansson et al. 2007; Crump and Trudeau 2009)
PCBs	*R. norvegicus*	Disruption to DA, glutamate, GABA, 5-HT and others.	(Fonnum and Mariussen 2009)
Pulp and paper mill effuents	*C. auratus*	Disruption of DA, GABA, glutamate systems	*In vitro* (Basu et al. 2009)
	Pimephales promelas	Differential gene expression in hypothalamus measured by microarray	100% effluent exposure, females (Popesku et al. 2010)

Note. DA, dopamine, 5-HT, serotonin, NA, noradrenaline, FSH, follicle-stimulating hormone, LH, luteinizing hormone, ACTH, adrenocorticotropic hormone, TSH, thyroid-stimulating hormone, GABA, gamma-aminobutyric acid.

changes, some of which will most certainly be maladaptive.

The hypothalamus–pituitary–gonad (HPG) axis tightly regulates vertebrate reproduction through the production of the gonadotropins, and much of the endocrine disruption research to date focuses on reproductive upsets, such as gonadal maturation and gametogenesis, sexual differentiation and behavior, or sex steroid mimics. Other regulatory axes, namely, the hypothalamus–pituitary–thyroid (HPT) and hypothalamus–pituitary–adrenal (HPA) axes, also contribute to reproductive regulation at various levels (including transcriptional, receptors, hormonal, or cellular) of "cross-talk." For example, hormones from the HPA axis modulate the activity of the HPG axis and vice versa (Dobson et al. 2003). The cross-talk or reciprocal regulation of the HPT and HPG axes during development is another good example (Hogan et al. 2007). Conceptually, the importance of this integrative endocrine communication is that a pollutant that mimics or disrupts a specific reproductive neuroendocrine pathway is also likely capable of affecting a stress- or thyroid-dependent neuroendocrine pathway. Therefore, cross-talk at all levels of a neuroendocrine axis needs to be taken into consideration when interpreting the recognized phenomenon of endocrine disruption.

Pharmaceuticals and Personal Care Products

Human and veterinary pharmaceutical usage results in the release of compounds to aquatic environments that are deliberately engineered to alter physiological states. Pharmaceuticals are eliminated from the body either in their original form or as by-products of the metabolic system. Drugs and their metabolites are ultimately flushed down the drain when eliminated from the body or disposed of improperly and end up in aquatic systems receiving municipal effluent. This exposes fish and other aquatic wildlife to pharmacologically active agents.

TABLE 4. Evidence of Neuroendocrine Disruption in Invertebrates

Disruptor	Species	Effect	Treatment
Cadmium	*Lymnaea palustris*	Disrupted calcium currents in nerve collar neurons	1 mg/L waterborne (Szucs et al. 1994)
	L. stagnalis	Inhibition GABA-activated chloride currents via increased calcium levels in nerve collar cells	In vitro, 50 μ*M* cell perfusion (Molnár et al. 2004)
	Uca pugilator	Blocking of NA-stimulated release of light-adapting hormone	10 mg/L waterborne (Reddy et al. 1997a)
		Inhibition of PDH synthesis	8.5 mg/kg injection (Reddy and Fingerman 1995)
	Procambarus clarkii	Increased release of GIH	1 mg/L waterborne (Rodríguez et al. 2000)
		Acetylcholinesterase inhibition	5 ppm waterborne (Devi and Fingerman 1995)
	Chasmagnathus granulata	Inhibited GIH release	0.5 mg/L waterborne (Medesani et al., 2004)
Municipal effluents	*Elliptio complanata*	Decreased 5-HT and DA, increased MAO activity in nerve ganglia	Injections and exposure to plume (Gagné and Blais, 2003)
		Decreased MAO and 5-HT transporter activity, increased DAT activity in nerve ganglia	Exposure to plume (Gagné and Blais, 2007)
		Increased DA, 5-HT, increased DAT, MAO, and COX activity, decreased 5-HT transporter activity	Direct exposure to aeration lagoon (Gagné and Blais, 2007)
		Decreased GABA, decreased GAD and MAO activity, increased 5-HT, DA, increased 5-HT transporter, DAT, and acetylcholinesterase activity; effects not mitigated by ozone treatment	Direct exposure to primary and ozone-treated effluents (Gagné et al., 2007)
PCBs	*U. pugilator*	Suppressed NA release from neural tissue, inhibiting PDH release from sinus gland	Injection of Aroclor 1242 (Hanumante et al. 1981)
Naphthalene	*U. pugilator*	Suppressed NA release from neural tissue, inhibiting PDH release from sinus gland	Injection (Staub and Fingerman 1984)
	P. clarkii	Suppression of GSH release	10 mg/L waterborne (Sarojini et al. 1994)
Copper	*Palaemon elegans*	CHH release and hyperglycemia, triggered by 5-HT stimulation	5 mg/L waterborne (Lorenzon et al., 2004; Lorenzon et al., 2005)
	C. granulata	Inhibited GIH release	0.1 mg/L waterborne (Medesani et al., 2004)
Mercury	*P. elegans*	CHH release and hyperglycemia	5 mg/L waterborne (Lorenzon et al., 2004)
	P. clarkii	Inhibition of 5-HT stimulated release of GSH	0.5 mg/kg injection (Reddy et al. 1997b)
		Acetylcholinesterase inhibition	0.2 ppm waterborne (Devi and Fingerman 1995)
Lead	*P. clarkii*	Acetylcholinesterase inhibition	100 ppm waterborne (Devi and Fingerman 1995)
Organophosphates and organocarbamates	*crustaceans*	Acetylcholinesterase inhibition	(Rapetto et al. 1988; Surendranath et al. 1990; Reddy et al. 1990)
Azadirachtin	*insects*	Blocked release of neurosecretory material, disruption of acetylcholine, GABA, and increased 5-HT	(Mordue and Blackwell 1993)
	Labidura riparia	Disruption of allatostatins, which inhibit JH synthesis	0.5, 1, 2, and 3 μg injection (Sayah et al. 1998)

Note. DA, dopamine, 5-HT, serotonin, NA, noradrenaline, FSH, follicle-stimulating hormone, LH, luteinizing hormone, ACTH, adrenocorticotropic hormone, TSH, thyroid-stimulating hormone, GABA, gamma-aminobutyric acid, PDH, pigment-dispersing hormone, GIH, gonad-inhibiting hormone, GSH, gonad-stimulating hormone, MAO, monoamine oxidase, DAT, dopamine transporter, COX, cyclooxygenase, CHH, crustacean hyperglycemic hormone, and JH, juvenile hormone.

Ethynylestradiol (EE2) is widely used for birth control and has been detected at appreciable levels (1–800 ng/L) in municipal effluents and waters receiving these effluents (Desbrow et al. 1998; Ternes et al. 1999; Kolpin et al. 2002). EE2 is neuroactive at environmentally relevant levels, being able to bind to and activate estrogen-response-elements (ERE) in the brains of vertebrates such as the frog, *Xenopus laevis,* and goldfish, *Carassius auratus* (Trudeau et al. 2005). Furthermore, environmentally relevant concentrations of EE2 induced disruption of neuroendocrine functions in adult and developing fish and frogs.

Waterborne EE2 exposures to newly hatched *X. tropicalis* tadpoles through to metamorphosis skewed the adult sex ratio toward female individuals at concentrations as low as 1 n*M*, producing defects in reproductive tissues of females. Decreased estrogen receptor alpha (ERα) expression occurred in the brain of the juveniles with these defects, suggesting that EE2 may interfere with the development of the reproductive system via reorganizations in the brain (Pettersson et al. 2006).

While environmentally relevant exposures of EE2 to male goldfish were shown to increase aromatase B expression (CYP19B) in the hypothalamus and telencephalon (Martyniuk et al. 2006a), microarray analysis performed with zebrafish telencephalon did not confirm this result, although differential expression of numerous other genes was observed in the liver and telencephalon (Martyniuk et al. 2007). Through microarray analysis of the brains of EE2- exposed male Japanese medaka (*Oryzias latipes*), significantly decreased expression of gonadotropin-releasing hormone (GnRH) receptor 1 (GnRH-R1) occurred at 5000 ng/L, which is a much higher concentration than one might find in the environment. Decreased androgen receptor alphaARα mRNA was observed at the more meaningful (in the context of environmental effects) concentration of 5 ng/L, and these males exhibited impaired sexual behavior (Zhang et al. 2008a). Changes in ARα in the brain of fish exposed to environmentally relevant levels of EE2 may have implications on processes such as steroidal feedback in the reproductive axis or disrupted spawning due to impaired reproductive behaviour.

The importance of the timing of exposure to neuroendocrine-disrupting chemicals is highlighted in studies where dietary (125 μg/g) (Shved et al. 2007) and waterborne (5 and 25 ng/L) (Shved et al. 2008) exposures of EE2 suppressed insulin-like growth factor 1 (IGF-1) in the brains of female tilapia (*Oreochromis niloticus*), but only at certain stages of development. IGF-1 and IGF-1 receptors are highly expressed in extrahepatic tissues (such as the brain) during ontogeny, suggesting it plays an important role during tissue differentiation, growth, and development (Perrot et al. 1999). In the waterborne exposure, ERα mRNA was determined, being elevated in male brains at 30 d postfertilization (DPF) but suppressed by the end of the experiment (100 DPF). In females, ERα was decreased over the course of the EE2 exposure (Shved et al. 2008).

Microarray analysis of the hypothalamic tissues of mature, prespawning female goldfish exposed to 50 μg/L of fadrozole, a potent aromatase inhibitor that suppresses serum estradiol (E2) and is used in the treatment of breast cancer in Japan, resulted in differential expression of many estrogen-responsive genes in the hypothalamus and telencephalon, including the decrease of aromatase B mRNA in both tissues (Zhang et al. 2009). The importance of aromatase B in the brain is now well known in fish (Diotel et al. 2010). Disruptions in brain aromatase may be indicative of disruption of neurogenesis, as it is only expressed in radial glial cells that give rise to neurons in fish brains (Diotel et al. 2010).

Clotrimazole is a chlorinated imidazole used by humans and animals as an antifungal treatment. Clotrimazole is present in municipal effluents and detectable downstream from waste water treatment plants at levels from <1 to 34 ng/L, and exposure of clotrimazole in the micrograms per liter range decreased aromatase activity in the brains of developing *X. tropicalis* during gonadal differentiation (Berg et al. 2009; Gyllenhammar et al. 2009). Ketoconazole, another imidazole-type

fungicide, is used in personal care products such as antidandruff shampoos and antifungal skin ointments. Waterborne ketoconazole (3–300 μl/L) has an inhibitory effect on fecundity in female Japanese medaka and decreased whole-brain GnRH receptor expression (Zhang et al. 2008b). This result is hard to interpret, because the role of GnRH receptors in the brain is not well known. However, functional assessments of pituitary sensitivity to GnRH-induced LH release are required to test the hypothesis that changes in receptors following environmentally relevant antifungal exposures are physiologically meaningful.

Selective serotonin reuptake inhibitors (SSRI) are used as antidepressants for their ability to inhibit serotonin reuptake by presynaptic cells, increasing serotonin (5-HT) concentrations in the synaptic cleft. In fish and mammals, there is evidence that 5-HT is important for neuroendocrine control of growth, feeding, reproduction, and stress. It is clear that SSRI are now considered serious aquatic environmental contaminants with neuroendocrine effects that disrupt feeding and reproduction (Mennigen et al. 2008; 2009; Oakes et al. 2010). However, more detail is provided in the review in this issue by Mennigen et al. (2011).

Studies on pharmaceuticals and personal care products as environmental neuroendocrine disruptors in the aquatic environment are still relatively new (Table 1). Popesku et al. (2008) started to analyze the effects of model pharmacological agents on hypothalamic function in fish. However, the neuroendocrine-disrupting potential of pharmaceuticals and personal care products released in municipal waste waters needs to be thoroughly examined, as many of these products are specifically designed to affect neurochemicals and enzymes that regulate brain function. As the effects of neuroactive compounds are investigated in model species and mechanisms of action are proposed for environmentally relevant concentrations, studies need to extend to field investigations to determine whether the effects are indeed observed in wild populations, as is the case with EE2 (Kidd et al. 2007). It is also noteworthy that neuroactive pharmaceuticals are only part of the problem. Neuroactive illicit drugs such as cocaine, ecstasy, or amphetamines and their metabolites are now found in aquatic environments (Zuccato and Castiglioni 2009; Metcalfe et al. 2010) and are likely to affect key neurotransmitter systems, regulative neuroendocrine function, and behavior in aquatic vertebrates as in humans.

Organochlorine (OC) Pesticides

The heavy use of pesticides in agriculture often results in chemicals being washed into aquatic systems and the soil by rain or entering the air by volatilization. Pesticides and their residues on plants destined for human or animal consumption expose us to toxic or neuroactive chemicals daily, while other, more persistent chemicals may end up in humans and wildlife through the process of biomagnification up food webs. These chemicals are designed with the express purpose of producing toxicity in pests or disrupting an organism's normal physiological state. Some examples of a certain class of pesticides, the organochlorines (OC) (Table 2), which have been notorious since the early days of the "green revolution" for their endocrine-disruptive effects, are presented.

Atrazine is a chlor-*s*-triazine herbicide that is extensively used globally despite being banned in the European Union. Goldman et al. (1999) reviewed the adverse effects of atrazine, specifically the inhibition of the rat luteinizing hormone (LH) preovulatory surge. Prolactin release from the pituitary was also suppressed by atrazine, and Goldman et al. (1999) indicated that these disruptive effects occur at the level of the brain rather than the pituitary. Ottinger et al. (2009) demonstrated the neuroendocrine effects of atrazine in birds, highlighting atrazine's potential to affect GnRH in the Japanese quail, *Coturnix japonica*.

Methoxychlor is an OC pesticide that was developed shortly after the ban of DDT (dichlorodiphenyltrichloroethane) and has largely replaced its use in agriculture. Methoxychlor has widely reported estrogenic

effects (Cummings 1997), and one study detected its presence in 1.2% of food samples in the United States (Duggan et al. 1983). Methoxychlor fed to female rats (25 mg/kg/d) was also shown to disrupt prolactin secretion through the inhibition of dopamine (DA), a potent inhibitor of prolactin (Lafuente et al. 2000a). Circulating LH was also lower in methoxychlor-treated rats (Lafuente et al. 2000a). Methoxychlor also increased noradrenaline (NA) and 5-HT in the anterior hypothalamus while decreasing 5-HT in the posterior hypothalamus in male rats fed at 25 mg/kg/d, providing further insight toward mechanisms by which methoxychlor may disrupt LH- and prolactin-mediated control of reproductive endpoints (Lafuente et al. 2008). Ottinger et al. (2005) showed that environmentally relevant dietary exposures of methoxychlor to adult Japanese quail enhanced gonadotropin-releasing hormone (GnRH) in the hypothalamus of 5 ppm treated males and 0.5 ppm treated females. When hypothalamic GnRH was measured in the young adult offspring of the treated parents, the female offspring of the 0.5 ppm treated group also had elevated GnRH, demonstrating the ability of endocrine disruptors to produce multigenerational changes to normal brain physiology. Ottinger et al. (2009) postulated that increased hypothalamic GnRH is due to inhibition of release of GnRH from this region as both NA and adrenaline, two catecholamines that stimulate GnRH release, were found to be reduced in the treated quail.

Methoxychlor was also found to alter the behavior of female mice, producing a defeminized or masculinized behavior upon prenatal exposure (Panzica et al. 2007). Panzica et al. (2007) measured D1-like receptor density in the nucleus accumbens and olfactory tubercle of prenatally exposed mice and attributed the altered behaviors to changes in brain reward pathways targeted by amphetamine, possibly involving monoaminergic circuits. Changes to neuroendocrine function or disruption of the endocrine axes might often manifest in behavioral changes. Panzica el al. (2007) extensively explored the neurological and behavioral effects of other xenoestrogens, and data on behavioral effects of endocrine disruptors are published in Palanza et al. (1999) and Ottinger et al. (2008).

Vinclozolin is a chlorinated dicarboximide fungicide that is used on food crops, golf courses and turf, and ornamental plants. When vinclozolin was fed (10 mg/kg/d) to female Dutch-belted rabbits (*Oryctolagus cuniculus*), the offspring had significantly fewer neurons containing GnRH in the anterior hypothalamus/preoptic area (AH/POA) and organum vasculosum of the lamina terminalis regions of the brain when compared to controls (Bisenius et al. 2006). A rise in calbindin-positive neurons was observed in the AH/POA in both male and female offspring. Calbindin plays an important role as a biomarker for sexual dimorphism, with females having more of these neurons than males in the sexually dimorphic region of the POA (Sickel and McCarthy 2000; Bisenius et al. 2006). Veeramachaneni et al. (2006) reported decreased follicle-stimulating hormone (FSH) secretion and inhibited sexual behavior in adult male rabbits born from females that were treated orally with vinclozolin (7.2 and 72 mg/kg/d) between gestation day 15 and postnatal week 4. These male offspring were less sensitive to GnRH-induced increases in both circulating FSH and testosterone (T). Injection of Japanese quail eggs with vinclozolin (25, 50, or 100 ppm) also resulted in disruption of GnRH and reproductive behavior, but these effects were only seen in males, with differential effects observed depending on the brain region and the dose used (McGary et al. 2001). However, in the same experiment (McGary et al. 2001), circulating sex steroids were unchanged in both male and female treatment groups.

Prochloraz is a chlorinated imidazole-type fungicide used in agriculture. Prochloraz residues were found in straw used as livestock fodder and detected in the milk and meat from goats that ingested this prochloraz-containing fodder (Campbell 1983). Prochloraz exerts inhibitory effects on reproduction, such as reducing fecundity and decreasing GnRH, GnRH receptors, and aromatase expression in

the brains of female Japanese medaka exposed to high (3–300 μg/L) waterborne concentrations (Zhang et al. 2008b).

Dieldrin is a cyclodiene pesticide that antagonizes the GABA-A type receptor and blocks Cl^- entry into neurons. Given the importance of GABA in the stimulatory control of LH release in fish (Trudeau et al. 1997), Martyniuk et al. (2010a) determined global transcriptomic and proteomic responses to dieldrin in the large mouth bass (*Micropterus salmoides*) hypothalamus. Martyniuk et al. (2010a) showed acute responses and concluded that impaired reproduction following dieldrin exposure may result in dieldrin-mediated neurotoxicity, upsets in LH, and potentially altered steroid receptor signalling. In another study Martyniuk et al. (2010b) fed bass with food contaminated with dieldrin. The mRNA levels for ER-beta mRNA levels were significantly lower in the hypothalamus after dieldrin feeding. Cluster and pathway analyses showed that genes and proteins involved in human neuropathologies such as Alzheimer's disease, inflammation, DNA damage, and ischemia were also affected by dietary dieldrin, as in the previous acute exposure study (Martyniuk et al. 2010a; 2010b).

Industrial Contaminants

Industrial processes, such as mining or the burning of fossil fuels, release naturally occurring toxic and neuroactive substances (Table 3) to the environment at levels much higher than what most life is able to adapt to. Toxic and endocrine-disrupting by-products created through the processing of materials and manufacturing of goods are released from smokestacks to the air and effluent pipes to water.

Cadmium (Cd) is an industrially important metal, being primarily released to the environment through the burning of fossil fuels and municipal wastes. Cadmium is a reproductive endocrine disruptor (U.S. EPA 2000) capable of bioaccumulating into the milligram per kilogram body weight range in both aquatic and terrestrial organisms (Frazier 1979; Wijnhoven et al. 2007). Cadmium is known to affect neurotransmitter levels in the brains of exposed organisms (Lafuente et al. 2001a). In a series of experiments, Cd exposure to rats was shown to affect DA, 5-HT, and NA in different brain regions, with the severity and direction of the impact depending on route, dose, and timing of exposure (Lafuente et al. 2000b; 2000c; 2001a; 2001b; 2003). Endpoints controlled by these neurochemicals (FSH, LH, adrenocorticotropic hormone [ACTH], prolactin, and thyroid-stimulating hormone [TSH]) were also differentially impacted depending on the difference between experimental designs, stressing the fact that predictions on how this metal, or any other neuroactive chemical, may impact wild populations depends on the route by which the population is exposed. Cadmium is also capable of upregulating GnRH expression in the whole brain of injected largemouth bass (Martyniuk et al. 2009).

When an organism is exposed to more than one contaminant at a time, which is most certainly the case in wild populations, the effects observed have the potential to be additive, synergistic, or antagonistic. Experiments performed by Pillai et al. (2003) determined the combined effects of intraperitoneal (ip) injections of either 0.5 mg/kg body weight of lead (Pb) and Cd alone or in combination in the hypothalamus of female adult rats. Pillai et al. (2003) found that Pb + Cd treatment abated reductions of 5-HT observed in the hypothalamus of Cd-only-treated rats. Dopamine levels were also decreased in Cd-treated, but not in Pb- or Pb + Cd-treated groups. Lead and Cd alone and in combination led to reduced NA. Decreased levels of both LH and FSH were observed in the Cd-treated group, while neither LH nor FSH was markedly changed in the Pb-exposed group. When Pb was administered with Cd, the effects were not significantly different from the Cd alone effect on LH and FSH. Pillai et al. (2003) compared and contrasted the effects of Pb and Cd seen in their experiments with those that also showed disruption of 5-HT, NA, and DA and the downstream effects on LH and FSH secretion.

Mercury (Hg) and its organic form, methylmercury (MeHg), are well known for their neurotoxicity (Ratcliffe et al 1996). The effects of mercury on neuroendocrine control of reproduction in fish were reviewed by Crump and Trudeau (2009) and effects on neurotransmitter systems were reviewed by Castoldi et al. (2001) and Johansson et al. (2007). While most studies occur in the laboratory setting using model animals with high-dose and acute exposures, chronic neuroendocrine disruption of the gamma-aminobutyric acid (GABA) system at environmentally relevant dietary concentrations (0.1–2 ppm for 3 mo) of MeHg was noted in the fish-eating captive juvenile male American mink (*Neovison vison*), supporting the notion that neuroendocrine disruption may indeed be occurring in wild populations of fish-eating animals (Basu et al. 2010).

Polychlorinated biphenyls (PCB) and brominated flame retardants also exert neurotoxic and neuroendocrine effects. The effects of PCB in the brains of rats are widespread, altering DA turnover and the uptake of glutamate and GABA, inhibiting GABA signalling, and decreasing 5-HT levels. Fonnum and Mariussen (2009) reviewed the neuroendocrine effects of PCB and brominated flame retardants.

NEUROENDOCRINE DISRUPTION IN INVERTEBRATES

While most investigations on EDC focused on effects in vertebrates, the importance of the impacts of EDC on invertebrates should not be overlooked. Invertebrates represent 95% of animal species and play an invaluable role in any ecosystem. Furthermore, the endocrinology of many invertebrates is well understood, and their sensitivity, size, ease of handling, fast generation time, and/or more facilitative regulatory restrictions make them convenient sentinels for monitoring ecosystem health. A few examples of neuroendocrine disruption in invertebrates are provided in Table 4. More extensive reviews on EDC in invertebrates are found in deFur (2004), Lagadic et al. (2007), and LeBlanc (2007).

Molluscs

In the review of endocrine disruption by Lagadic et al. (2007), data demonstrated how Cd was capable of affecting embryo development, numbers of egg masses, number of eggs per egg mass, and egg hatching in different species of the genus *Lymnaea*, an aquatic pulmonate gastropod. Szücs et al. (1994) showed that chronic exposure to waterborne Cd (1 mg/L) increased calcium currents in nerve collar neurons of *L. stagnalis*. Collar cells were found to stimulate spermatogenesis in gastropods (Takeda 1982). An in vitro study in *L. stagnalis* by Molnár et al. (2004) also suggested that Cd inhibited GABA-activated chloride currents by increasing intracellular calcium levels in collar cells. These are plausible mechanisms by which Cd may disrupt neurotransmitter control of reproduction in gastropods.

The effects of municipal effluents on the reproductive axis in the freshwater mussel, *Elliptio complanata*, were assessed by Gagné and Blaise (2003). In mussels, the gonads are surrounded with nerve ganglia that project into the gonad and control spawning and gonadal maturation through DA, 5-HT, and arachidonic acid cyclooxygenase (COX) activity (Gagné and Blaise 2003; Gagné et al. 2007a). Briefly, COX synthesizes prostaglandin and induces 5-HT release, which stimulates spawning, and 5-HT stimulates, while DA inhibits, gonadal maturation. 5-HT also plays an important role in sex determination, specifically in the differentiation of male sex organs. Extracts of municipal effluents exerted an estrogen-like response upon injection or direct exposure to a municipal effluent plume (Gagné and Blaise 2003): Effluent exposure and E2 injection decreased 5-HT and DA levels in nerve tissues. Different effluents produced either an elevation, such as observed upon E2 injections (Gagné and Blaise 2003), or a decrease in monoamine oxidase (MAO) activity (Gagné et al. 2007a). Gagné et al. (2007a) also measured 5-HT and DA transporters, demonstrating that exposure to the plume decreased 5-HT transport and increased DA transport. Gagne et al. (2007a) concluded that these effluents exerted an

overall stimulatory effect on reproduction, yet this hypothesis was not directly tested. When the mussels were put directly in the aeration lagoons that were used to treat the effluents, DA and 5-HT levels, as well as DA transport, MAO activity, and COX activity levels, were increased, while 5-HT transport levels were decreased. Data suggested that conflicting results might be produced by higher levels and diversity of neuroactive pharmaceuticals present in the lagoon versus the plume, where advanced degradation and dilution may occur. Ozone treatment of effluents was unable to mitigate effects produced by primary effluents (reduced GABA levels and glutamic acid decarboxylase [GAD] and MAO activity, and elevated 5-HT, DA, and 5-HT transporter, DA transporter, and acetylcholinesterase activity), demonstrating that certain treatments of effluents may be ineffective at removing neuroactive chemicals from municipal wastes (Gagné et al. 2007b).

Crustaceans

Neuroendocrine disruption was also described in crustaceans (Fingerman et al. 1998). Injections of the PCB mixture Aroclor 1242 (Hanumante et al. 1981) and the polyaromatic hydrocarbon (PAH) naphthalene (Staub and Fingerman 1984) are capable of inhibiting NA-stimulated release of pigment-dispersing hormone (PDH) in the fiddler crab, *Uca pugilator*. The PDH octadecapeptide is released from the sinus gland and modulates color change in crustaceans. Waterborne exposures of Cd (10 mg/L) block the stimulatory action of NA on light-adapting hormone release, reducing the eye's ability to adapt to light conditions (Reddy et al. 1997a). There is also evidence that injected Cd (8.5 mg/kg body weight injection) inhibits PDH synthesis in the fiddler crab (Reddy and Fingerman 1995).

Fingerman et al. (1998) summarized experiments on the effects of Cd, DDT, sumithion (an organophosphate insecticide), and naphthalene on hyperglycemia in different crustaceans. Evidence indicated modulation of hyperglycemia was attributed to changes in production and release of crustacean hyperglycemic hormone (CHH). The CHH family of polypeptide neurohormones plays important roles in carbohydrate and lipid metabolism (Zarubin et al. 2009). In the shrimp *Palaemon elegans*, lethal levels (5 mg/L) of both copper (Cu)- and Hg-contaminated seawaters induced a rapid release of CHH from the sinus gland in the eyestalk and a subsequent hyperglycemic response (Lorenzon et al. 2004). At sublethal levels, 0.1 mg/L Cu and Hg produced a slower release of CHH, with recovery occurring after 3 h. The sublethal intermediate level of 0.5 mg/L Hg was capable of eliciting an even stronger release of CHH than the lethal dose of 5 mg/L. In a follow-up study, Lorenzon et al. (2005) determined that CHH release by Cu was triggered by release of 5-HT in the eyestalk, which subsequently stimulated CHH secretion.

In the crayfish *Procambarus clarkii*, the stimulatory effect of 5-HT, which triggers gonad-stimulating hormone (GSH) release from the brain and thoracic ganglia (Sarojini et al. 1995), on in vivo ovarian maturation was inhibited by 0.5 mg/kg body weight injections of Hg (Reddy et al. 1997b). Ovarian maturation was also inhibited in *P. clarkii* by waterborne exposure of naphthalene at 10 mg/L (Sarojini et al. 1994). The inhibitory effect of naphthalene on ovarian maturation may have been via inhibition of GSH release from neural tissues (Sarojini et al. 1994). Waterborne Cd (1 mg/L) was also found to enhance secretion of gonad-inhibiting hormone (GIH) from the eyestalks of the fiddler crab, *U. pugilator*, which led to inhibition of gonadal growth either through suppression of GSH release and/or direct inhibition of the oocytes (Rodríguez et al. 2000). Conversely, in the crab *Chasmagnathus granulata*, both waterborne Cu (0.1 mg/L) and Cd (0.5 mg/L) were found to inhibit GIH release (Medesani et al. 2004), highlighting the possible existence of species-specific response to Cd exposure.

Acetylcholinesterase (AChE) activity in neural tissues was shown to be inhibited by Hg, Cd, and Pb in *P. clarkii* (Devi and Fingerman 1995).

AChE inhibition was also observed in crustaceans after exposure to organophosphates (Repetto et al. 1988; Surendranath et al. 1990) and organocarbamates (Rao et al. 1991), pesticides specifically designed to inhibit AChE in insect pests. The inhibitory effects on AChE activity in the prawn *Metapenaeus monoceros* by the organocarbamate carbaryl were still evident after a 10-d recovery period where the prawns were kept in clean water. Inhibition of AChE was stronger in prawns exposed to commercial-grade versus technical-grade carbaryl, indicating emulsifiers or other ingredients in the pesticide mix increased the penetrability of carbaryl in tissues and cells (Reddy et al. 1990).

Insects

Many insecticidal products are manufactured with the express purpose of interfering with the hormonal systems in pest insects. The intended use and modes of actions of these pesticides are beyond the scope of this review, although the potential exists for neuroendocrine disruption in nontarget insects. One example of a potential neuroendocrine disruptor in nontarget insects is azadirachtin (AZA), an insecticidal limonoid isolated from the seeds of the neem tree, *Azadirachta indica*, which has antifeeding, growth-regulating, and sterilizing properties in insects. AZA is capable of blocking the release of neurosecretory material from the corpus cardiaca, interacting with the acetylcholinergic and GABAergic systems in the brain–ring gland complexes, and elevating levels of 5-HT in the brain, subesophageal ganglion, and corpus cardiaca in some insects (Mordue and Blackwell 1993). AZA was also shown to inhibit vitellogenesis in the earwig *Labidura riparia*, by altering the distribution of the neuropeptide allatostatins (Sayah et al. 1998). These allatostatins inhibit juvenile hormone (JH) synthesis in the corpora allata. Juvenile hormone controls growth and development of immature insects and vitellogenesis in adults. In this study, treatment of earwigs with JH counteracted the inhibitory effects of AZA on earwig vitellogenesis.

THE IMPORTANCE OF AN INTEGRATIVE, SYSTEMS APPROACH TO UNDERSTAND ENDOCRINE DISRUPTION

Endocrine disruption research, especially in vertebrate models, has been largely biased toward the hypothesis that pollutants may or may not be estrogenic EDC. Estrogen receptor binding, in vitro transcriptional reporter gene assays, and vitellogenin production are all indicative of essentially one pathway. Only a small proportion of the hundreds of published studies on EDC in the last decade or so reflect neuroendocrine disruption processes. One case where relatively narrow hypothesis testing strategies have only partially solved a major problem in environmental biology is that of the effects of pulp and paper mill effluent (PPME) on fish reproduction. This serves as a good example where the broader approach of systems biology and integrative physiology is warranted.

Since 1994, the Canadian Fisheries Act requires pulp and paper mills to conduct environmental effects monitoring (EEM) to determine whether PPME affect fish, and thereby determine whether regulations adequately protect the environment on a site-specific basis. Many studies show that PPME rapidly inhibit egg production in fish, and that life-cycle exposure alters sex ratio and induces intersex animals (males with eggs in their testes) (Hewitt et al. 2006; McMaster et al. 2006; Parrott et al. 2006; Hewitt et al. 2008). An important pattern to emerge from these studies is that despite significant reductions in acute effluent toxicity, suspended solids, and released chlorinated organics, PPME still reduces gonad size and inhibits reproduction in fish. The minimal reproductive improvements observed over numerous cycles of EEM (Hewitt et al. 2008) generated a cause-and-effect hypothesis that EDC in effluents are functioning as steroid mimics or interfere with steroid signaling. Bioassays have been based largely on assessments of gonadal function via in vivo and in vitro measurements of biologically active sex steroids in response to effluent exposures,

and studies have assessed whether fish tissue, effluent, and wood extracts contain ligands for fish sex steroid receptors (Hewitt et al. 2006; 2008). Pioneering work by MacLatchy and Van Der Kraak (1995) indicated that the phytoestrogen β-sitosterol is one possible anti-reproductive chemical. These studies are all-important, and there is strong evidence that compounds in effluents are acting as steroid mimics or interfering with steroid signaling, but they have yet to fully explain the observed reproductive effects downstream from Canadian mills. It is clear from national surveys that other pathways may be implicated in reproductive disruption, as metabolic disruption was also noted in affected populations (Hewitt et al. 2006; 2008).

The observation that PPME rapidly and reversibly inhibit spawning in the fathead minnow (Kovacs et al. 2007) was our first important clue that disruption of neuroendocrine processes may be involved. Given that ovulation and sperm release are essentially triggered by neural events (i.e., it is a neuroendocrine cascade), it was postulated that PPME contain neuroactive chemicals. Only one early study attempted to assess the neuroendocrine status of fish exposed to effluents. Van Der Kraak et al. (1992) demonstrated that in a white sucker, *Catostomus commersonii*, population downstream from a bleached kraft mill, LH levels were suppressed and pituitary LH responsiveness to GnRH was reduced. Changes in the neuroendocrine control of gonadotropin secretion may provide one mechanism by which pulp mill effluents exert adverse effects.

The first study to explore the neuroactivity potential of pulp mill effluent was published by Basu et al. (2009). It is well known that the catecholamine DA is a potent inhibitor of GnRH and LH release in multiple teleost species (Dufour et al. 2005). This inhibition is so potent that it is necessary to co-inject DA receptor antagonists and GnRH agonists to induce ovulation in many fish species. Another key neurotransmitter system controlling LH release is the amino acid gamma-aminobutyric acid (GABA). GABA is synthesized from glutamic acid by the enzyme glutamic acid decarboxylase. By inhibition of DA and stimulation of GnRH, GABA stimulates LH release in goldfish (Trudeau 1997; Popesku et al. 2008). Basu et al. (2009) found that PPME contain compounds that interact with key enzymes and receptors that regulate the GABAergic, glutamatergic, and DAergic modulation of GnRH and LH in fish. Further validation of the neuroendocrine disruption hypothesis for the effects of PPME on fish was obtained using microarray analysis coupled with comparative genomic approaches. Expression profiling of the female fathead minnow hypothalamus revealed distinct effects of various PPME (Popesku et al. 2010). Comparison of these effects in the fathead minnow with the effects of specific DA agonists injected into female goldfish indicated that PPME, to some extent, may be acting on DA-sensitive pathways, thereby inhibiting reproduction. PPME in receiving waters, as with all situations of environmental contamination, represent complex mixtures of diverse substances with endocrine-disrupting activities. It is likely that there are effects through a multitude of mechanisms and several levels of the neuroendocrine system. Therefore, it is essential that a more comprehensive approach to the problem of neuroendocrine disruption be taken.

CONCLUSIONS AND FUTURE DIRECTION

We used several examples to illustrate that diverse environmental pollutants have the potential to disrupt neuroendocrine control mechanisms. One major observation is that negative effects of diverse pollutants on reproductive processes are common. This has been used by some sectors as an argument against the specificity of effects of EDC, as they may represent a nonspecific stress response. This critique has little scientific basis. Rather, reproductive effects reflect the complexity of neuroendocrine control mechanisms, and chemicals inhibit reproduction specifically through multiple highly complex pathways. For example, SSRI disturb neuroendocrine control of

reproduction through the central 5-HT system (Mennigen et al. 2008; 2011), but EE2 specifically alters neuroendocrine control of reproduction via activation of multiple nuclear estrogen receptors (ER) in numerous estrogen-sensitive neuronal systems, in addition to specific ER-mediated effects in gonads and liver (Martyniuk et al. 2006b; Zhang and Trudeau 2006). The multitude of neuropeptides, neurotransmitters, enzymes, hormones, and receptors that may be involved in physiological responses to neuroendocrine disruptors is staggering, and perhaps this has dissuaded some from exploring the neuroendocrine effects of endocrine disruptors. Another important challenge is separating observed effects on the neuroendocrine system as a whole from direct actions of EDC on the central nervous system. For example, one may observe an effect on a receptor or peptide in the hypothalamus, but is this change a direct result of exposure to a pollutant or the consequence of effects of EDC action on a peripheral hormone involved in a feedback loop? This reveals the major challenge, and one of the most compelling reasons to adopt the methods of systems biology and integrative physiology to address the effects of EDC. Another major challenge is the testing of the neuroendocrine disruption hypothesis at the scale of the ecosystem. This will require a concerted effort between experts in vertebrate and invertebrate neuroendocrinology, ecotoxicology, and ecology. For example, many assays for neurotransmitter/neuropeptide receptor binding or for neurotransmitter enzyme synthesis and degradation are well established and could be more broadly used for screening environmental samples obtained from areas where animals, including humans, are being exposed to pollutants.

Neuroendocrine disruptors are defined as pollutants in the environment that are capable of acting as agonists/antagonists or altering the synthesis and/or metabolism of neuropeptides, neurotransmitters, or neurohormones, which subsequently alter diverse physiological, behavioral, or hormonal processes to affect an animal's capacity to reproduce, develop and grow, or deal with stress and other challenges. By adopting a definition of neuroendocrine disruption that encompasses both direct physiological targets and their indirect downstream effects, from the level of the individual to the ecosystem, a comprehensive picture of the consequences of EDC exposure may emerge.

REFERENCES

Basu, N., Ta, C. A., Waye, A., Mao, J., Hewitt, M., Arnason, J. T., and Trudeau, V. L. 2009. Pulp and paper mill effluents contain neuroactive substances that potentially disrupt neuroendocrine control of fish reproduction. *Environ. Sci. Technol*. 43:1635–1641.

Basu, N., Scheuhammer, A. M., Rouvinen-Watt, K., Evans, R. D., Trudeau, V. L., and Chan, L. H. M. 2010. In vitro and whole animal evidence that methylmercury disrupts GABAergic systems in discrete brain regions in captive mink. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol*. 151:379–385.

Berg, C., Gyllenhammar, I., and Kvarnryd, M. 2009. *Xenopus tropicalis* as a test system for developmental and reproductive toxicity. *J. Toxicol. Environ. Health A* 72: 219–225.

Bisenius, E. S., Veeramachaneni, D. N. R., Sammonds, G. E., and Tobet, S. 2006. Sex differences and the development of the rabbit brain: Effects of vinclozolin. *Biol. Reprod*. 75:469–476.

Bourguignon, J. P., Rasier, G., Lebrethon, M. C., Gérard, A., Naveau, E., and Parent, A. S. 2010. Neuroendocrine disruption of pubertal timing and interactions between homeostasis of reproduction and energy balance. *Mol. Cell. Endocrinol*. 324:110–120.

Campbell, J. K. 1983. *Residues of prochloraz in milk and tissues of a lacting goat fed straw containing residues of radiactive prochloraz*. FBC report METAB/83/8 submitted to FAO by FBC Ltd. Unpublished.

Castoldi, A. F., Coccini, T., Ceccatelli, S., and Manzo, L. 2001. Neurotoxicity and

molecular effects of methylmercury. *Brain. Res. Bull*. 55:197–203.

Colborn, T., vom Saal, F. S., and Soto, A. M. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect*. 101:378–384.

Cooper, R. L., Goldman, J. M., and Stoker, T. E. 1999. Neuroendocrine and reproductive effects of contemporary-use pesticides. *Toxicol. Ind. Health* 15:26–36.

Crump, D., Lean, D., Trudeau, V. L. 2002. Octylphenol and UV-B radiation alter larval development and hypothalamic gene expression in the leopard frog (*Rana pipiens*). *Environ. Health Perspect*. 110:277–284.

Crump, K. L., and Trudeau, V. L. 2009. Mercury-induced reproductive impairment in fish. *Environ. Toxicol. Chem*. 28:895–907.

Cummings, A. M. 1997. Methoxychlor as a model for environmental estrogens. *Crit. Rev. Toxicol*. 27:367–379.

deFur, P. L. 2004. Use and role of invertebrate models in endocrine disruptor research and testing. *Inst. Lab. Anim. Res. J*. 45:484–493.

Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P., and Waldock, M. 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ. Sci. Technol*. 32:1549–1558.

Devi, M., and Fingerman, M. 1995. Inhibition of acetylcholinesterase activity in the central nervous system of the red swamp crayfish, *Procambarus clarkii*, by mercury, cadmium, and lead. *Bull. Environ. Contam. Toxicol*. 55:746–750.

Diotel, N., Page, Y. L., Mouriec, K., Tong, S.-K., Pellegrini, E., Vaillant, C., Anglade, I., Brion, F., Pakdel, F., Chung, B., and Kah, O. 2010. Aromatase in the brain of teleost fish: Expression, regulation and putative functions. *Front. Neuroendocrinol*. 31:172–192.

Dobson, H., Ghuman, S., Prabhakar, S., and Smith, R. 2003. A conceptual model of the influence of stress on female reproduction. *Reproduction* 125:151–163.

Dufour, S., Weltzien, F.-A., Sebert, M.-E., Le Belle, N., Vidal, B., Vernier, P., and Pasqualini, C. 2005. Dopaminergic inhibition of reproduction in teleost fishes: Ecophysiological and evolutionary implications. *Ann. NY Acad. Sci*. 1040:9–21.

Duggan, R. E., Corneliussen, P. E., Duggan, M. G., McMahon, B. M., and Martin, R. J. 1983. *Pesticide residue levels in foods in the United States from 1 July 1969 to 10 June 1976*. Washington, DC: Food and Drug Administration and Association of Official Analytical Chemistry.

Fingerman, M., Jackson, N. C., and Nagabhushanam, R. 1998. Hormonally-regulated functions in crustaceans as biomarkers of environmental pollution. *Comp. Biochem. Physiol. C Pharmacol. Toxicol, Endocrinol*. 120:343–350.

Fonnum, F., and Mariussen, E. 2009. Mechanisms involved in the neurotoxic effects of environmental toxicants such as polychlorinated biphenyls and brominated flame retardants. *J. Neurochem*. 111:1327–1347.

Frazier, J. M. 1979. Bioaccumulation of cadmium in marine organisms. *Environ. Health Perspect*. 28:75–79.

Gagné, F., and Blaise, C. 2003. Effects of municipal effluents on serotonin and dopamine levels in the freshwater mussel *Elliptio complanata*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol*. 136:117–125.

Gagné, F., Blaise, C., André, C., Gagnon, C., and Salazar, M. 2007a. Neuroendocrine disruption and health effects in *Elliptio complanata* mussels exposed to aeration lagoons for wastewater treatment. *Chemosphere* 68:731–743.

Gagné, F., Cejka, P., André, C., Hausler, R., and Blaise, C. 2007b. Neurotoxicological effects of a primary and ozonated treated wastewater on freshwater mussels exposed to an experimental flow-through system. *Comp. Biochem. Physiol. C Toxicol. Pharmacol*. 146:460–470.

Goldman, J. M., Stoker, T. E., and Cooper, R. L. 1999. Neuroendocrine and reproductive effects of contemporary-use pesticides. *Toxicol. Ind. Health* 15:26–36.

Gore, A. C. 2008. Neuroendocrine systems as targets for environmental

endocrine-disrupting chemicals. *Fertil. Steril*. 89:101–102.

Gore, A. C. 2010. Neuroendocrine targets of endocrine disruptors. *Hormones* 9:16–27.

Gore, A. C. and Patisaul, H. B. 2010. Neuroendocrine disruption: Historical roots, current progress, questions for the future. *Front. Neuroendocrinol*. 31:395–399.

Gyllenhammar, I., Eriksson, H., Söderqvist, A., Lindberg, R. H., Fick, J., and Berg, C. 2009. Clotrimazole exposure modulates aromatase activity in gonads and brain during gonadal differentiation in *Xenopus tropicalis* frogs. *Aquat. Toxicol*. 91:102–09.

Hanumante, M. M., Fingerman, S. W., and Fingerman, M. 1981. Antagonism of the inhibitory effect of the polychlorinated biphenyl preparation, Aroclor 1242, on color changes of the fiddler crab, *Uca pugilator*, by norepinephrine and drugs affecting noradrenergic neurotransmission. *Bull. Environ. Contamin. Toxicol*. 26:479–484.

Hewitt, L. M., Parrott, J. L., and McMaster, M. E. 2006. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: Sources and characteristics of bioactive substances. *J. Toxicol. Environ. Health B* 9:341–356.

Hewitt, L. M., Kovacs, T. G., Dube, M. G., MacLatchy, D. L., Martel, P. H., McMaster, M. E., Paice, M. G., Parrott, J. L., van den Heuvel, M. R., and van der Kraak, G. J. 2008. Altered reproduction in fish exposed to pulp and paper mill effluents: Roles of individual compounds and mill operating conditions. *Environ. Toxicol. Chem*. 27:682–697.

Hogan, N. S., Crump, K. L., Duarte, P., Lean, D. R. S., and Trudeau, V. L. 2007. Hormone cross-regulation in the tadpole brain: Developmental expression profiles and effect of T3 exposure on thyroid hormone- and estrogen-responsive genes in *Rana pipiens*. *Gen. Comp. Endocrinol*. 154:5–15.

Hotchkiss, A. K., Rider, C. V., Blystone, C. R., Wilson, V. S., Hartig, P. C., Ankley, G. T., Foster, P. M., Gray, C. L., and Gray, L. E. 2008. Fifteen years after "Wingspread"—Environmental endocrine disrupters and human and wildlife health: Where we are today and where we need to go. *Toxicol. Sci*. 105:235–259.

Johansson, C., Castoldi, A., Onishchenko, N., Manzo, L., Vahter, M., and Ceccatelli, S. 2007. Neurobehavioural and molecular changes induced by methylmercury exposure during development. *Neurotoxicol. Res*. 11:241–260.

Khan, I. A., and Thomas, P. 2001. Disruption of neuroendocrine control of luteinizing hormone secretion by Aroclor 1254 involves inhibition of hypothalamic tryptophan hydroxylase activity. *Biol. Reprod*. 64:955–964.

Kidd, K. A., Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M., and Flick, R. W. 2007. Collapse of a fish population after exposure to a synthetic estrogen. *Proc. Natl. Acad. Sci. USA* 104:8897–8901.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., and Buxton, H. T. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: A national reconnaissance. *Environ. Sci. Technol*. 36:1202–1211.

Kovacs, T. G., Martel, P. H., and Ricci, M. 2007. A shortened adult fathead minnow reproduction test developed for investigation of cause and investigation of solution work involving pulp and paper mill effluents. *Water Qual. Res. J. Can*. 42:91–100.

Lafuente, A., Márquez, N., Pousada, Y., Pazo, D., and Esquifino, A. I. 2000a. Possible estrogenic and/or antiandrogenic effects of methoxychlor on prolactin release in male rats. *Arch. Toxicol*. 74:270–275.

Lafuente, A., Márquez, N., Pazo, D., and Esquifino, A. 2000b. Effects of subchronic alternating cadmium exposure on dopamine turnover and plasma levels of prolactin, GH and ACTH. *Biometals* 13:47–55.

Lafuente, A., Márquez, N., Pérez-Lorenzo, M., Pazo, D., and Esquifino, A. I. 2000c. Pubertal and postpubertal cadmium exposure

differentially affects the hypothalamic-pituitary-testicular axis function in the rat. *Food Chem. Toxicol*. 38:913–923.

Lafuente, A., Marquez, N., Perez-Lorenzo, M., Pazo, D., and Esquifino, A. I. 2001a. Cadmium effects on hypothalamic-pituitary-testicular axis in male rats. *Exp. Biol. Med*. 226:605–611.

Lafuente, A., Marquez, N., Pazo, D., and Esquifino, A. I. 2001b. Cadmium effects on dopamine turnover and plasma levels of prolactin, GH and ACTH. *J. Physiol. Biochem*. 57:231–236.

Lafuente, A., Cano, P., and Esquifino, A. I. 2003. Are cadmium effects on plasma gonadotropins, prolactin, ACTH, GH and TSH levels, dose-dependent? *BioMetals* 16:243–250.

Lafuente, A., Cabaleiro, T., Caride, A., and Esquifino, A. I. 2008. Toxic effects of methoxychlor administered subcutaneously on the hypothalamic-pituitary-testicular axis in adult rats. *Food Chem. Toxicol*. 46:1570–1575.

Lagadic, L., Coutellec, M.-A., and Caquet, T. 2007. Endocrine disruption in aquatic pulmonate molluscs: Few evidences, many challenges. *Ecotoxicology* 16:45–59.

LeBlanc, G. 2007. Crustacean endocrine toxicology: A review. *Ecotoxicology* 16:61–81.

Lorenzon, S., Edomi, P., Giulianini, P. G., Mettulio, R., and Ferrero, E. A. 2004. Variation of crustacean hyperglycemic hormone (cHH) level in the eyestalk and haemolymph of the shrimp *Palaemon elegans* following stress. *J. Exp. Biol*. 207:4205–4213.

Lorenzon, S., Edomi, P., Giulianini, P. G., Mettulio, R., and Ferrero, E. A. 2005. Role of biogenic amines and cHH in the crustacean hyperglycemic stress response. *J. Exp. Biol*. 208:3341–3347.

MacLatchy, D. L., and Van Der Kraak, G. J. 1995. The phytoestrogen β-sitosterol alters the reproductive endocrine status of goldfish. *Toxicol. Appl.* 134:305–312.

Martyniuk, C. J., Xiong, H., Crump, Sardana, R., Nadler, A., Gerrie, E. R., Xia, X., and Trudeau, V. L. 2006a. Gene expression profiling in the neuroendocrine brain of male goldfish (*Carassius auratus*) exposed to 17□-ethinylestradiol. *Physiol. Genom*. 27:328–336.

Martyniuk, C., Marlatt, V., Gallant, N., Wiens, S., Woodhouse, M., and Trudeau, V. L. 2006b. Current perspectives on estrogen action and estrogen receptors in teleost fishes. In *Fish endocrinology*, ed. M. Reineke, G. Zaccone, and B.G. Kapoor, 625–664. Enfield, NH: Science Publishers.

Martyniuk, C. J., Gerrie, E. R., Popesku, J. T., Ekker, M., and Trudeau, V. L. 2007. Microarray analysis in the zebrafish (*Danio rerio*) liver and telencephalon after exposure to low concentration of 17alpha-ethinylestradiol. *Aquat. Toxicol*. 84:38–49.

Martyniuk, C. J., Sanchez, B. C., Szabo, N. J., Denslow, N. D., and Sepúlveda, M. S. 2009. Aquatic contaminants alter genes involved in neurotransmitter synthesis and gonadotropin release in largemouth bass. *Aquat. Toxicol*. 95:1–9.

Martyniuk, C. J., Feswick, A., Spade, D. J., Kroll, K. J., Barber, D. S., and Denslow, N.D. 2010a. Effects of acute dieldrin exposure on neurotransmitters and global gene transcription in largemouth bass (*Micropterus salmoides*) hypothalamus. *Neurotoxicology* 31:356–366.

Martyniuk, C. J., Kroll, K. J., Doperalski, N. J., Barber, D. S., and Denslow, N. D. 2010b. Genomic and proteomic responses to environmentally relevant exposures to dieldrin: Indicators of neurodegeneration? *Toxicol. Sci*. 117:190–199.

McGary, S., Henry, P. F. P., and Ann Ottinger, M. 2001. Impact of vinclozolin on reproductive behavior and endocrinology in Japanese quail (*Coturnix coturnix japonica*). *Environ. Toxicol. Chem*. 20:2487–2493.

McMaster, M. E., Hewitt, L. M., and Parrott, J. L. 2006. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: field studies and mechanistic research. *J. Toxicol. Environ. Health B* 9:319–339.

Medesani, D. A., Greco, L. S. L., and Rodríguez, E. M. 2004. Interference of cadmium and copper with the endocrine control of ovarian growth, in the estuarine crab *Chasmagnathus granulata*. *Aquat. Toxicol*. 69:165–174.

Mennigen, J. A., Martyniuk, C. J., Crump, K., Xiong, H., Zhao, E., Popesku, J., Anisman, H., Cossins, A. R., Xia, X., and Trudeau, V. L. 2008. Effects of fluoxetine on the reproductive axis of female goldfish (*Carassius auratus*). *Physiol. Genom*. 35:273–282.

Mennigen, J. A., Harris, E. A., Chang, J. P., Moon, T. W., and Trudeau, V. L. 2009. Fluoxetine affects weight gain and expression of feeding peptides in the female goldfish brain. *Regul. Peptides* 155:99–104.

Mennigen, J. A., Stroud, P., Zamora, J. M., Moon, T. W., and Trudeau, V. L. 2011 Pharmaceuticals as neuroendocrine disruptors: Lessons learned from fish on Prozac. *J. Environ. Toxicol. Health B*. 14:387–412.

Metcalfe, C. D., Tindale, K., Li, H., Rodayan, A., and Yargeau, V. 2010. Illicit drugs in Canadian municipal wastewater and estimates of community drug use. *Environ. Pollut*. 158:3179–3185.

Molnár, G., Salánki, J., and Kiss, T. 2004. Cadmium inhibits GABA-activated ion currents by increasing intracellular calcium level in snail neurons. *Brain Res*. 1008:205–211.

Mordue, A. J., and Blackwell, A. 1993. Azadirachtin: An update. *J. Insect Physiol*. 39:903–24.

Oakes, K. D., Coors, A., Escher, B. I., Fenner, K., Garric, J., Gust, M., Knacker, T., Küster, A., Kussatz, C., Metcalfe, C. D., Monteiro, S., Moon, T. W., Mennigen, J. A., Parrott, J., Péry, A. R., Ramil, M., Roennefahrt, I., Tarazona, J. V., Sánchez-Argüello, P., Ternes, T. A., Trudeau, V. L., Boucard, T., Van Der Kraak, G. J., and Servos, M. R. 2010. Environmental risk assessment for the serotonin re-uptake inhibitor fluoxetine: Case study using the European risk assessment framework. *Integr. Environ. Assess. Manage*. 6:524–539.

Ottinger, M. A., Wu, J. M., Hazelton, J. L., Abdelnabi, M. A., Thompson, N., Quinn, J. M. L., Donoghue, D., Schenck, F., Ruscio, M., Beavers, J., and Jaber, M. 2005. Assessing the consequences of the pesticide methoxychlor: neuroendocrine and behavioral measures as indicators of biological impact of an estrogenic environmental chemical. *Brain Res. Bull*. 65:199–209.

Ottinger, M. A., Lavoie, E., Thompson, N., Barton, A., Whitehouse, K., Barton, M., Abdelnabi, M., Quinn Jr, M. J., Panzica, G. C., and Viglietti-Panzica, C. 2008. Neuroendocrine and behavioral effects of embryonic exposure to endocrine disrupting chemicals in birds. *Brain Res. Rev*. 57:376–385.

Ottinger, M. A., Lavoie, E. T., Thompson, N., Bohannon, M., Dean, K., and Quinn, M. J., Jr. 2009. Is the gonadotropin releasing hormone system vulnerable to endocrine disruption in birds? *Gen. Comp. Endocrinol*. 163:104–108.

Palanza, P., Morellini, F., Parmigiani, S., and vom Saal, F. S. 1999. Prenatal exposure to endocrine disrupting chemicals: Effects on behavioral development. *Neurosci. Biobehav. Rev*. 23:1011–1027.

Panzica, G. C., Viglietti-Panzica, C., Mura, E., Quinn, M. J., Jr., Lavoie, E., Palanza, P., and Ottinger, M. A. 2007. Effects of xenoextrogens on the differentiation of behaviorally-relevant neural circuits. *Front. Neuroendocrinol*. 28:179–200.

Parrott, J. L., McMaster, M. E., and Hewitt, L. M. 2006. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: Development and application of fish bioassays. *J. Toxicol. Environ. Health B* 9:297–317.

Perrot, V., Moiseeva, E. B., Gozes, Y., Chan, S. J., Ingleton, P., and Funkenstein, B. 1999. Ontogeny of the insulin-like growth factor system (IGF-I, IGF-II, and IGF-1R) in gilthead seabream (*Sparus aurata*): Expression and cellular localization. *Gen. Comp. Endocrinol*. 116:445–460.

Pettersson, I., Arukwe, A., Lundstedt-Enkel, K., Mortensen, A. S., and Berg, C. 2006.

Persistent sex-reversal and oviducal agenesis in adult *Xenopus* (Silurana) *tropicalis* frogs following larval exposure to the environmental pollutant ethynylestradiol. *Aquat. Toxicol*. 79:356–365.

Pillai, A., Priya, L., and Gupta, S. 2003. Effects of combined exposure to lead and cadmium on the hypothalamic-pituitary axis function in proestrous rats. *Food Chem. Toxicol*. 41:379–384.

Popesku, J. T., Martyniuk, C. J., Mennigen, J., Xiong, H., Zhang, D., Xia, X., Cossins, A. R., and Trudeau, V. L. 2008. The goldfish (*Carassius auratus*) as a model for neuroendocrine signaling. *Mol. Cell. Endocrinol*. 293:43–56.

Popesku, J. T., Tan, E. Y. Z., Martel, P. H., Kovacs, T. G., Rowan-Carroll, A., Williams, A., Yauk, C., and Trudeau, V. L. 2010. Gene expression profiling of the fathead minnow (*Pimephales promelas*) neuroendocrine brain in response to pulp and paper mill effluents. *Aquat. Toxicol*. 99:379–388.

Porte, C., Janer, G., Lorusso, L. C., Ortiz-Zarragoitia, M., Cajaraville, M. P., Fossi, M. C., and Canesi, L. 2006. Endocrine disruptors in marine organisms: Approaches and perspectives. *Comp. Biochem. Physiol. C Toxicol. Pharmacol*. 143:303–315.

Rao, K. V., Ghouselazam, S., and Surendranath, P. 1991. Inhibition and recovery of selected target enzyme activities in tissues of penaeid prawn, *Metapenaeus monoceros* (Fabricius), exposed to different insecticides. *Indian J. Exp. Biol*. 29:489–491.

Ratcliffe, H.E., Swanson, G. M., and Fischer, L.J. 1996. Human exposure to mercury: A critical assessment of the evidence of adverse effects. *J. Toxicol. Environ. Health* 49: 221–270.

Reddy, M. S., Jayaprada, P., and Rao, K. V. 1990. Recovery of carbaryl inhibited AChE in penaeid prawn, *Metapenaeus monoceros*. *Biochem. Int*. 22:189–198.

Reddy, P. S., and Fingerman, M. 1995. Effect of cadmium chloride on physiological color changes of the fiddler crab, *Uca pugilator*. *Ecotoxicol. Environ. Saf*. 31:69–75.

Reddy, P. S., Nguyen, L. K., Obih, P., and Fingerman, M. 1997a. Effect of cadmium chloride on the distal retinal pigment cells of the fiddler crab, *Uca pugilator*. *Bull. Environ. Contam. Toxicol*. 58:504–510.

Reddy, P. S., Tuberty, S. R., and Fingerman, M. 1997b. Effects of cadmium and mercury on ovarian maturation in the red swamp crayfish, *Procambarus clarkii*. *Ecotoxicol. Environ. Safety* 37:62–65.

Repetto, G., Sanz, P., and Repetto, M. 1988. In vivo and in vitro effect of triclorfon on esterases of the red crayfish *Procambarus clarkii*. *Bull. Environ. Contam. Toxicol*. 41:597–603.

Rodríguez, E. M., López Greco, L. S., and Fingerman, M. 2000. Inhibition of ovarian growth by cadmium in the fiddler crab, *Uca pugilator* (Decapoda, Ocypodidae). *Ecotoxicol. Environ. Safety* 46:202–206.

Sarojini, R., Nagabhushanam, R., and Fingerman, M. 1994. A possible neurotransmitter-neuroendocrine mechanism in naphthalene-induced atresia of the ovary of the red swamp crayfish, *Procambarus clarkii*. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol*. 108:33–38.

Sarojini, R., Nagabhushanam, R., and Fingerman, M. 1995. Mode of action of the neurotransmitter 5-hydroxytryptamine in stimulating ovarian maturation in the red swamp crayfish, *Procambarus clarkii*: An in vivo and in vitro study. *J. Exp. Zool*. 271:395–400.

Sayah, F., Idaomar, M., Soranzo, L., and Karlinsky, A. 1998. Endocrine and neuroendocrine effects of Azadirachtin in adult females of the earwig *Labidura riparia*. *Tissue Cell* 30:86–94.

Shved, N., Berishvili, G., Baroiller, J.-F., Segner, H., and Reinecke, M. 2008. Environmentally relevant concentrations of 17β-ethinylestradiol (EE2) interfere with the growth hormone (GH)/insulin-like growth factor (IGF)-I system in developing bony fish. *Toxicol. Sci*. 106:93–102.

Shved, N., Berishvili, G., D'Cotta, H., Baroiller, J.-F., Segner, H., Eppler, E., and Reinecke, M.

2007. Ethinylestradiol differentially interferes with IGF-I in liver and extrahepatic sites during development of male and female bony fish. *J. Endocrinol*. 195:513–523.

Sickel, M. J., and McCarthy, M. M. 2000. Calbindin-D28k immunoreactivity is a marker for a subdivision of the sexually dimorphic nucleus of the preoptic area of the rat: Developmental profile and gonadal steroid modulation. *J. Neuroendocrinol*. 12:397–402.

Singh, H., and Singh, T. P. 1980. Short-term effect of two pesticides on the survival, ovarian 32P uptake and gonadotrophic potency in a freshwater catfish, *Heteropneustes fossilis* (Bloch). *J. Endocrinol*. 85:193–199.

Smith, C. G. 1983. Reproductive toxicity: hypothalamic-pituitary mechanisms. *Am. J. Ind. Med*. 4:107–112.

Staub, G. C., and Fingerman, M. 1984. A mechanism of action for the inhibition of black pigment dispersion in the fiddler crab, *Uca pugilator*, by naphthalene. *Comp. Biochem. Physiol. C Toxicol. Pharmacol*. 79:447–453.

Surendranath, P., Lazam, S., and Rao, V. R. 1990. Monitoring insecticide biodegradation in penaeid prawn *Penaeus indicus* following subacute exposure to phosphamidon and DDT through reclamation studies. *Environ. Ecol*. 8:397–400.

Szücs, A., Salánki, J., and S.-Rózsa, K. 1994. Effects of chronic exposure to cadmium- or lead-enriched environments on ionic currents of identified neurons in *Lymnaea stagnalis* L. *Cell. Mol. Neurobiol*. 14:769–780.

Takeda, N. 1982. Source of the tentacular hormone in terrestrial pulmonates. *Cell. Mol. Life Sci*. 38:1058–1060.

Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R. D., and Servos, M. 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants—I. Investigations in Germany, Canada and Brazil. *Sci. Total Environ*. 225:81–90.

Trudeau, V. L. 1997. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Carassius auratus*. *Rev. Reprod*. 2:55–68.

Trudeau, V. L., Chiu, S., Kennedy, S. W., and Brooks, R. J. 2002. Octylphenol (OP) alters the expression of members of the amyloid protein family in the hypothalamus of the snapping turtle, *Chelydra serpentina serpentina*. *Environ. Health Perspect*. 110:269–275.

Trudeau, V. L., Turque, N., Le Mevel, S., Alliot, C., Gallant, N., Coen, L., Pakdel, F., and Demeneix, B. 2005. Assessment of estrogenic endocrine-disrupting chemical actions in the brain using in vivo somatic gene transfer. *Environ. Health Perspect*. 113:329–334.

U.S. Environmental Protection Agency. 2000. *Cadmium compounds* (online). Available at: http://www.epa.gov/ttn/atw/hlthef/cadmium.html (accessed June 30, 2010).

U.S. Environmental Protection Agency. 2007. *What are endocrine disruptors?* Available at: http://www.epa.gov/endo/pubs/edspoverview/whatare.htm (accessed July 30, 2010).

Van Der Kraak, G. J., Munkittrick, K. R., McMaster, M. E., Portt, C. B., and Chang, J. P. 1992. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. *Toxicol. Appl. Pharmacol*. 115:224–233.

van der Ven, K., Keil, D., Moens, L. N., Van Leemput, K., van Remortel, P., and De Coen, W. M. 2006. Neuropharmaceuticals in the environment: mianserin-induced neuroendocrine disruption in zebrafish (*Danio rerio*) using cDNA microarrays. *Environ. Toxicol. Chem*. 25: 2645–2652.

Veeramachaneni, D. N. R., Palmer, J. S., Amann, R. P., Kane, C. M., Higuchi, T. T., and Pau, K.-Y. F. 2006. Disruption of sexual function, FSH secretion, and spermiogenesis in rabbits following developmental exposure to vinclozolin, a fungicide. *Reproduction* 131:805–816.

Vos, J. G., Dybing, E., Greim, H. A., Ladefoged, O., Lambre, C., Tarazona, J. V., Brandt, I., and Vethaak, A. D. 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to

the European situation. *Crit. Rev. Toxicol*. 30:71–133.

Wijnhoven, S., Leuven, R. S., van der Velde, G., Jungheim, G., Koelemij, E. I., de Vries, F. T., Eijsackers, H. J., and Smits, A. J. 2007. Heavy-metal concentrations in small mammals from a diffusely polluted floodplain: Importance of species- and location-specific characteristics. *Arch. Environ. Contam. Toxicol*. 52:603–613.

Zarubin, T., Chang, E., and Mykles, D. 2009. Expression of recombinant eyestalk crustacean hyperglycemic hormone from the tropical land crab, *Gecarcinus lateralis*, that inhibits Y-organ ecdysteroidogenesis in vitro. *Mol. Biol. Rep*. 36: 1231–1237.

Zhang, D., and Trudeau, V. L. 2006. Integration of membrane and nuclear estrogen receptor signalling. *Comp. Biochem. Physiol. A Mol. Integr. Physiol*. 144:306–315.

Zhang, D., Popesku, J. T., Martyniuk, C. J., Xiong, H., Duarte-Guterman, P., Yao, L., Xia, X., and Trudeau, V. L. 2009. Profiling neuroendocrine gene expression changes following fadrozole-induced estrogen decline in the female goldfish. *Physiol. Genom*. 38:351–361.

Zhang, X., Hecker, M., Park, J.-W., Tompsett, A. R., Newsted, J., Nakayama, K., Jones, P. D., Au, D., Kong, R., Wu, R. S. S., and Giesy, J. P. 2008a. Real-time PCR array to study effects of chemicals on the hypothalamic–pituitary–gonadal axis of the Japanese medaka. *Aquat. Toxicol*. 88:173–182.

Zhang, X., Hecker, M., Jones, P. D., Newsted, J., Au, D., Kong, R., Wu, R. S. S., and Giesy, J. P. 2008b. Responses of the medaka HPG axis PCR array and reproduction to prochloraz and ketoconazole. *Environ. Sci. Technol*. 42:6762–6769.

Zoeller, R. T. 2008. Environmental neuroendocrine and thyroid disruption: relevance for reproductive medicine? *Fertil. Steril*. 89:99–100.

Zuccato, E., and Castiglioni, S. 2009. Illicit drugs in the environment. *Philos. Trans. R. Soc. A* 367:3965–3978.

Appendix 2

Environ. Sci. Technol. **2009,** *43,* 1635–1641

Pulp and Paper Mill Effluents Contain Neuroactive Substances That Potentially Disrupt Neuroendocrine Control of Fish Reproduction

NILADRI BASU,[†] CHIEU ANH TA,[‡] ANDREW WAYE,[‡] JINQIN MAO,[‡] MARK HEWITT,[§] JOHN T. ARNASON,[‡] AND VANCE L. TRUDEAU*,[‡]

Department of Environmental Health Sciences, University of Michigan School of Public Health, Ann Arbor, Michigan 48109, Department of Biology, University of Ottawa, Ottawa, ON, K1N 6N5, Canada, and National Water Research Institute, Environment Canada, Burlington, ON, L7R 4A6, Canada

Received September 9, 2008. Revised manuscript received November 15, 2008. Accepted November 19, 2008.

Here we show for the first time that components of pulp and paper mill effluents contain neuroactive substances that may impair fish reproduction. Grab samples of primary and secondary effluent were obtained from a representative pulp and paper mill in Eastern Canada. Effluents were fractionated using classic polarity and polyphenolic extraction methods into solvents of selected polarities (water, ethanol, ethyl acetate, and hexane). By means of in vitro, competitive assays on goldfish (*Carassius auratus*) brain tissues, the extracts were screened for their ability to interact with enzymes and receptors involved in gamma-aminobutyric acid (GABA), dopamine, glutamate, and acetylcholine-dependent neurotransmission. These neurotransmission pathways have essential regulatory roles in fish reproduction. Radioligand binding to the following neurotransmitter receptors were significantly impacted following in vitro incubations with extracts (percentage change from controls indicated in brackets): dopamine-2 (D2; 21−48% increase), GABA(A) receptor binding (65−67% decrease and 189% increase), *N*-methyl-D-aspartic acid (NMDA; 26−75% decrease), and muscarinic cholinergic (mACh; 42% increase). Activities of the following neurotransmitter-related enzymes were significantly impacted: monoamine oxidase (MAO; 14−48% decrease), GABA-transaminase activity (33% decrease and 21−69% increase), and acetylcholinesterase (AChE; 21−50% decrease). No changes in glutamic acid decarboxylase (GAD) activity were detected. These findings provide a novel and plausible mechanism by which pulp and paper mills effluents impair fish reproduction by interacting with neurotransmitter systems. Further work is required to identify the active compounds and explore whether these changes occur in vivo.

* Corresponding author tel.: +1 613-562-5800 ext 6165; fax: +1 613-562-5486; e-mail: trudeauv@uottawa.ca.
[†] University of Michigan School of Public Health.
[‡] University of Ottawa.
[§] Environment Canada.

Introduction

Over the past 30 years numerous studies have documented that fish residing downstream of pulp and paper mills suffer from impaired reproduction and fertility (*1*−*5*). Commonly observed symptoms have included reductions in gonad size, egg production, sex hormone levels, secondary sexual characteristics, and fecundity. Despite intense research efforts, the precise mechanisms underlying these adverse reproductive outcomes are not clear and the identification of bioactive substances have proven challenging. Bioassays in the past have been based largely on assessments of reproductive function via in vivo and in vitro measurements of biologically active sex steroids in response to effluent exposures. Most of these studies have focused on estrogenic (*6, 7*) and androgenic (*8*−*10*) pathways. While there is certainly strong evidence that these pathways are affected, they are not the only pathways that could be involved in the reproductive effects caused by pulp and paper mill effluents. In fact, many effluents have strong antireproductive activities but are neither strictly estrogenic nor androgenic in standard assays (*6*).

Although fundamental to our understanding of antireproductive effects (mainly at the level of the gonad or liver), previous studies concerning pulp and paper mill effluent have not experimentally considered that vertebrate reproduction is controlled by the brain through a tightly regulated hypothalamus−pituitary−gonad (HPG) communication axis (*11, 12*). In response to hormonal and environmental cues, the hypothalamus in the brain synthesizes and releases gonadotropin-releasing hormone (GnRH), which in turn stimulates the release of luteinizing hormone (LH) from the anterior pituitary. It is LH that regulates vertebrate fertility by controlling gonadal development, sex steroid production, ovulation, and sperm release.

The neural control of GnRH and LH is multifactorial, involving a multitude of classical neurotransmitters and sex steroids (*11*). In particular, through multiple mechanisms, dopamine is the main inhibitor of LH release in teleosts. Manipulative experiments have shown that dopamine binds to type 2 dopamine (D2) receptors in the pituitary gonadotrophs to inhibit LH release and impair fish reproduction (*13*−*15*). Conversely, gamma-aminobutyric acid (GABA) has a positive effect on fish reproduction as it stimulates the release of gonadotropin-releasing hormone (GnRH) and LH and inhibits dopamine turnover (*16*−*18*). Glutamate, the main excitatory neurotransmitter in the vertebrate brain, has a stimulatory effect on GnRH and LH release in fish (*19*). Blockage of glutamate signaling disrupts levels of sex hormones, delays the onset of puberty, and alters reproductive behavior in mammals (*20, 21*). Other studies using mouse GT1−7 cells have shown that activation of cholinergic receptors stimulates GnRH release in vitro (*22*).

While it is clear that fish reproduction is controlled by several neurotransmitter systems, the effects of pulp and paper mill effluents on these neural systems have yet to be explored in any vertebrate species. Here we hypothesized that bioactive compound(s) within pulp and paper mill effluents can interact with, and disrupt, various neurotransmitter receptors and enzymes important to fish reproduction. In this investigation, grab samples of primary- and secondary-treated effluents from a representative newsprint mill were fractionated using two established approaches (solvent series and polyphenolic extraction). The resulting extracts were subsequently screened for their ability to interact with key enzymes and receptors involved in the neurotransmission

10.1021/es802552m CCC: $40.75
Published on Web 01/14/2009

TABLE 1. Yields of Pulp and Paper Mill Effluent Extracts Obtained Using Solvent Series and Polyphenolic Extraction Methods

extraction method	solvent fraction	yield of primary effluent (mg/L)	yield of secondary effluent (mg/L)
solvent series	hexane	12	10
	EtOAc	200	30
	water	2,600	2,720
polyphenolic	EtOH	150	90
	water	2,180	2,000

of GABA (GABA(A) receptor binding, glutamic acid decarboxylase (GAD) activity, GABA-transaminase (GABA-T) activity), dopamine (dopamine-2 (D2) receptor binding, monoamine oxidase (MAO) activity), glutamate (*N*-methyl-D-aspartic acid (NMDA) receptor binding), and acetylcholine (muscarinic acetylcholine (mAch) receptor binding, acetylcholinesterase (AChE) activity) by means of in vitro, competitive assays using goldfish (*Carassius auratus*) brain tissues. Goldfish are an excellent model for studying vertebrate neuroendocrine signaling (*23*).

Experimental Methods

Pulp and Paper Mill Effluent Collections. One-liter grab samples each of primary- and secondary-treated effluents were collected at a representative Eastern Canadian mill. This mill uses thermomechanical pulping (TMP) to process spruce and fir chips into newsprint. The effluent from the mill is first subjected to primary treatment in a clarifier and then it is subjected to secondary treatment in a conventional activated sludge treatment plant. Test effluents were stored at 4 °C until they were processed and fractionated between February 1 and May 1, 2007.

Fractionation of Effluents. Four different extracts were collected using two established methods of extraction (classic polarity (*24*) and polyphenolic extraction (*25*)) and several solvents (hexane, ethyl acetate (EtOAc), ethanol (EtOH)), and water) (Table 1).

a. Solvent Series. Effluent (1 L) was extracted sequentially with 500 and 250 mL of hexane with 5 min gentle rocking in a separatory funnel to avoid foaming. The hexane extracts were combined and rotary evaporated at 30 °C to dryness, then collected, weighed, and stored at −20 °C. The residual water was then extracted with 500 and 250 mL of EtOAc in a separatory funnel. The EtOAc extracts were combined and rotary evaporated (30 °C) to dryness, then collected, weighed, and stored at −20 °C. The remaining water fraction was freeze-dried. All yields are reported (Table 1).

b. Polyphenolic Extraction. Polyvinylpolypyrrolidone (PVPP) powder (Sigma, St. Louis, MO) was added to effluents (1 L) in an Erlenmeyer flask at a ratio of 2.5 g/L. Samples were shaken overnight (200 rpm) and vacuum filtered through Whatman No. 1 paper. The resulting water fraction was freeze-dried, weighed, and stored at −20 °C. The PVPP filtrates were extracted with 2 × 200 mL of EtOH. The resulting EtOH extracts were collected, evaporated, freeze-dried, weighed, and stored at −20 °C. All yields are reported (Table 1).

As the purpose of this study was to determine if neuroactive components exist in pulp and paper mill effluents, all dried extracts were bioassayed at 0.5 mg/mL (except for the GAD assay, see below). As extraction yields were highly variable (Table 1), testing a fixed concentration of sample enabled direct comparisons to be made among all extracts. In order to relate 0.5 mg/mL to an equivalent volume of initial liquid effluent, the concentration tested (i.e., 0.5 mg/mL) can be divided by the yield (from Table 2). As an example,

TABLE 2. Methodological Aspects for Neurochemical Receptor Binding Assays

neurochemical receptor	buffer[a]	pretreatment of microplates[b]	radioligand, specific activity[c]	dissplacer information[d]	incubation condition
dopamine-2 (D2)	50 mM Tris HCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$	100 μL of 0.1% polyethylenimine	[3H]-spiperone, 15.7 Ci/mmol, 5 nM	(+)-butaclamol	90 min, 25 °C
GABA(A)	50 mM Tris HCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$	100 μL of 0.1% polyethylenimine	[3H]-muscimol, 18 Ci/mmol, 32 nM	muscimol	30 min, 4 °C
NMDA	50 mM Tris, 100 μM glycine, 100 μM L-glutamic acid	100 μL of indicated Tris buffer	[3H]-MK801, 22 Ci/mmol, 5 nM	MK-801	120 min, 25 °C
mAChR	50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl	100 μL of indicated Na/K buffer	[3H]-QNB, 42 Ci/mmol, 1 nM	atropine sulfate	60 min, 25 °C

[a] Buffers were adjusted to pH 7.4. [b] Plates were preconditioned for 30 min with 100 μL of buffer or 0.1% polyethylenimine (PE). [c] Radioligands were purchased from NEN/Perkin-Elmer (Boston, MA). [d] Displacers were tested at 100 μM.

bioassaying 0.5 mg/mL of the extract derived from the primary effluent using solvent series (yield = 2000 mg/L) was equivalent to testing 0.19 mL of the original effluent/mL.

Preparation of Goldfish Brain Tissues. The in vitro experiments were carried out on whole brain pooled from approximately 100 common goldfish (*Carassius auratus*). For receptor binding studies, cellular membranes were prepared by homogenizing tissues in 1:10 volumes of buffer (see Table 2 for specific details) according to established methods (*26*). The homogenate was centrifuged (32,000*g*, 15 min, 4 °C) and the resulting pellet was washed twice under the same conditions. The final pellet was resuspended in buffer and aliquots of cellular membranes were immediately frozen to −80 °C until required. For MAO and AChE assays, brain tissues were sonicated for 30 s in cold Na/K buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4) that included 0.5% (v/v) Triton X-100. Following a 10 min centrifugation at 15,000*g* (4 °C), the supernatant was removed and stored at −80 °C until required. For GAD assays, tissues were homogenized in 1:10 (w/v) of phosphate buffer (pH 7.5). The homogenate was stored at −80 °C until required. For GABA-T assays, brains were homogenized in 1:10 buffer (20% glycerol, 0.13% Triton X-100, 0.1 mM reduced glutathione, 0.1 mM pyridoxal-5′-phosphate, 1 mM Na_2EDTA, 10 mM K_2HPO_4, pH 6.8), and centrifuged at 1,500*g* (4 °C, 30 min). The resulting supernatant was stored at −80 °C until required. The concentration of protein in all samples was determined using the method of Bradford.

Neurotransmitter Receptor Binding Assays. For all receptor binding assays, 30 μg of membrane preparation was resuspended in 100 μL of buffer (see Table 2 for specific details) and added to microplate wells containing a 1.0 μM GF/B glass filter (Millipore, Boston, MA) according to established methods (*26*−*28*). Prior to the addition of radioligands (Table 2), samples were preincubated with effluent extracts (final concentration = 0.5 mg/mL) for 30 min. All assays were carried out under gentle agitation and reactions were terminated by vacuum filtration. The filters were rinsed three times with buffer and then allowed to soak for 96 h in 25 μL of OptiPhase Supermix Cocktail (Perkin-Elmer). Radioactivity retained by the filter was quantified by liquid scintillation counting in a microplate detector (Wallac Microbeta, Perkin-Elmer). Specific binding to the receptors was defined as the difference in radioligand bound in the presence and absence of 100 μM unlabeled displacer (Table 2). Percentage inhibition (expressed as "% of control binding") was determined by calculating specific binding in the presence and absence of extract. All samples were assayed in triplicate. Each assay run plate included test samples, as well as negative (blanks and solvent) and positive (displacer) controls.

Neurotransmitter Enzyme Assays. The activities of AChE and MAO were measured using 96-well microplates according to published methods (*26, 29*) that are briefly outlined here. For AChE activity, 0.5 μg of supernatant protein was mixed with 100 μM 10-acetyl-3,7-dihydroxyphenoxazine, 200 mU horseradish peroxidase, 20 mU choline oxidase, and 100 μM acetylcholine. For MAO activity, 5 μg of protein was mixed with 100 μM 10-acetyl-3,7-dihydroxyphenoxazine, 200 mU horseradish peroxidase, and 100 mM tyramine. Following a 30 min incubation period for both assays, the production of resorufin (λ_{ex} = 540, λ_{em} = 590) was monitored between 30 and 60 min (CytoFluor 2350, Millipore, Bedford, MA).

For GAD activity, 50 μg of brain homogenate was added to glass tubes containing 10 mM phosphate buffer (including 60 μM pyridoxal-5′-phosphate and 120 μM dithiothreitol, pH 7.4) according to published methods (*30*). The reaction was initiated by addition of 30 mM glutamate containing 0.1 μCi l-[1-^{14}C]glutamic acid (60.0 mCi/mmol; Amersham, Buckinghamshire, England) and a suspended Whatman GF/B filter paper soaked in Scintigest (Fisher Scientific, Ottawa, Canada). Following a 45 min reaction at 37 °C, the reaction was terminated by the addition of 0.25 M HCl. The vials were allowed to incubate for another 60 min. Radioactivity trapped on the filter paper was determined using a scintillation counter (Beckman Coulter LS6500 Multipurpose Scintillation Counter).

For GABA-T activity, homogenates were incubated in 100 mM potassium pyrophosphate buffer (containing 5 mM α-ketoglutarate, 4 mM NAD, 3.5 mM 2-mercaptoethanol, 10 μM pyridoxal-5′-phosphate, pH 8.6) for 15 min at 37 °C according to published methods (*30*). Following the addition of 10 mM GABA, absorbance was immediately monitored at 340 nM every 10 s for 2 min and the maximal velocity (i.e., slope) of the enzymatic reaction (V_{max}) was calculated.

For all enzyme assays, samples were preincubated with effluent extracts (final concentration = 0.5 mg/mL; except for GAD activity which was tested using a final effluent concentration of 0.05 mg/mL) for 30 min. Percentage inhibition (expressed as "% of control activity") was determined by calculating enzyme activity in the presence and absence of effluent extract. The effect of solvent carrier was tested in each assay. All samples were assayed in triplicate.

Statistical Analyses. All statistical operations were performed with Sigma Stat (Version 2.03, SPSS Inc., San Rafael, CA) using $\alpha = 0.05$ as the level of statistical significance. All results were normally distributed as determined by Bartlett's test. The inhibitory effects of extracts (compared to nonexposed and solvent controls) were assessed by means of *t* tests and ANOVAs (Tukey's post hoc).

Results and Discussion

Here we show that constituents in pulp and paper mill effluent contain neuroactive substances that, in vitro, directly interact with several neurotransmitter receptors and enzymes that are important regulators of fish reproduction. A high-throughput, biologically based, in vitro screening platform permitted us to rapidly assess the interactions between different types of effluent extracts and several neurochemical receptors and enzymes. Solvent controls did not significantly alter any of the measured parameters, and intrasample variation was less than 17% except for [3H]-muscimol binding to the GABA(A) receptor (22% variation).

From the primary effluent, extracts of water and EtOAc significantly potentiated [3H]-spiperone binding to D2 receptors, while only the water extract of the secondary effluent showed potentiation (Figure 1). The hexane extracts of both primary and secondary effluents showed no effects on D2 receptor binding. Solvent extracts derived from PVPP yielded differential responses as D2 receptor binding was significantly potentiated by the ethanol fraction from the primary effluent and by the water fraction from the secondary effluent. Dopamine is a catecholamine neurotransmitter that exerts a negative effect on fish reproduction. Activation of D2 receptors as observed here with effluent extracts (Figure 1) has the potential to disrupt fish reproduction by inhibiting the release of LH (*15*). The bioactive component(s) responsible for these potentiating effects were present in polar extracts from both primary and secondary effluents and this may facilitate compound isolation and identification in follow-up studies. Investigations on several plant species have shown that botanicals contain potent modulators of dopaminergic neurotransmission (*32, 33*). It is possible that pulp and paper mill effluents contain various phytochemicals that can potentiate dopamine function and inhibit LH release, and thus disrupt reproduction in fish. The fact that the polar extracts of primary and secondary effluent exhibited this activity suggests that these substances would be bioavailable

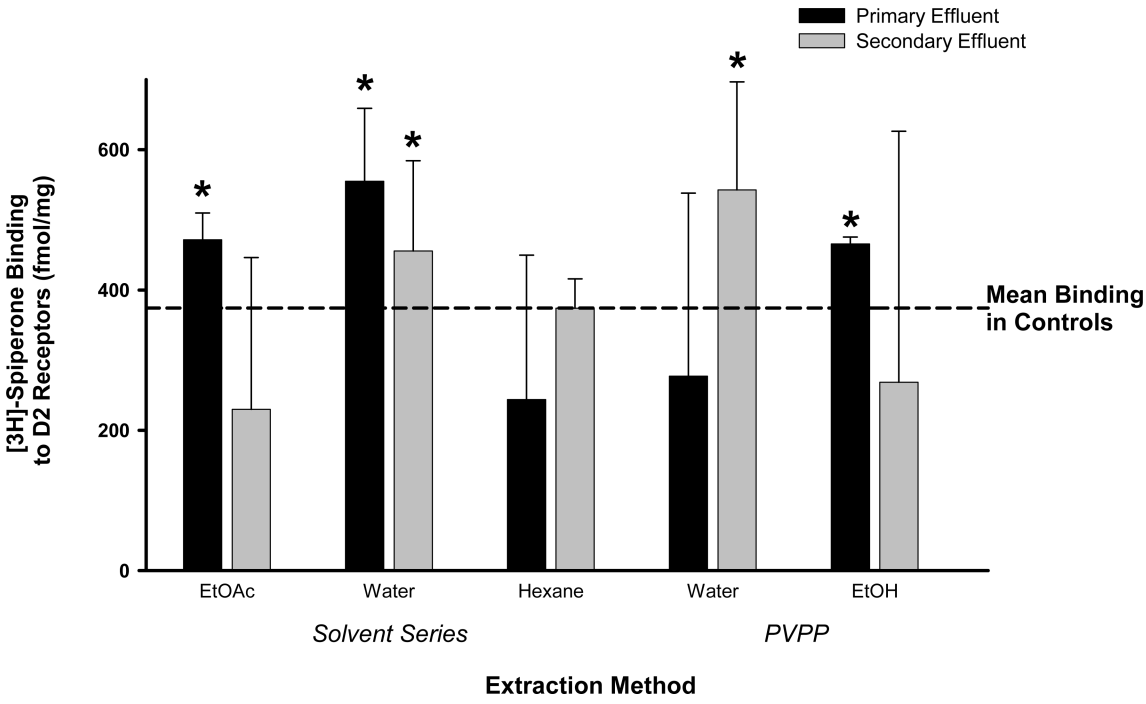


FIGURE 1. In vitro effects of pulp and paper mill effluent extracts on the binding of [^{3}H]-spiperone to the dopamine-2 (D2) receptor in the goldfish brain. Asterisks (*) represent significant differences ($p < 0.05$) in binding from control (nonexposed) samples (control binding is indicated as a dashed line). Bars represent mean (±SEM) from 3 assay runs.

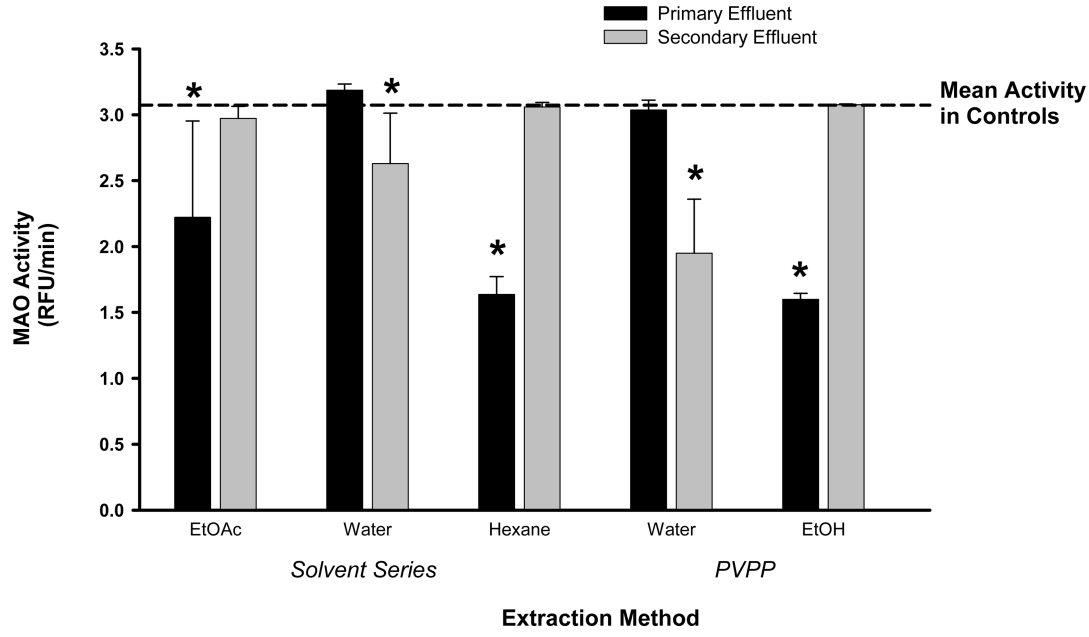


FIGURE 2. In vitro effects of pulp and paper mill effluent extracts on the activity of monoamine oxidase (MAO) in the goldfish brain. Asterisks (*) represent significant differences ($p < 0.05$) in enzyme activity from control (nonexposed) samples (control activity is indicated as a dashed line). Bars represent mean (±SEM) from 3 assay runs.

and could elicit their impacts rapidly in fish via a waterborne exposure. This is consistent with the body of evidence that shows the onset of inhibitory effects on such end points as circulating sex steroids (*34*) and egg production (*35*) in fish exposed to effluents occurs rapidly.

Dopamine is catabolized by the oxidative enzyme MAO. Here we found several extracts significantly inhibited MAO activity by nearly 50% of controls (Figure 2). In the primary effluent, enzyme inhibition was caused by extracts that were derived using organic solvents. Conversely, in the secondary effluent, enzyme inhibition was only caused by water-derived extracts. Reduced metabolic activity of MAO would result in the accumulation of synaptic dopamine. In concert with the aforementioned D2 receptor binding results, these findings further suggest that components in pulp and paper mill effluent can possibly impede fish reproduction by promoting dopaminergic neurotransmission. In vivo inhibition of MAO inhibits LH release in goldfish (*36*). Studies on relevant aquatic pollutants, including polychlorinated biphenyls (*37*) and cadmium (*38*), have shown that modulation of dopamine homeostasis by chemical stressors impairs reproductive success in fish.

In contrast to the antireproductive effects of dopamine, GABA stimulates LH release in fish. Our results suggest that component(s) within pulp and paper mill effluent can both inhibit and potentiate ligand binding to the GABA(A) receptor

TABLE 3. Summary of In Vitro Neurochemical Results, Expressed As a % of Control (Non-Exposed) Samples (For a Given Neurochemical Parameter, Asterisks (*) Represent Significant Differences ($p < 0.05$) from Controls)

		primary effluent					secondary effluent				
		solvent series extraction method			PVPP extraction method		solvent series extraction method			PVPP extraction method	
		ethyl acetate	water	hexane	water	ethanol	ethyl acetate	water	hexane	water	ethanol
neurochemical receptors	D2	126.0*	148.3*	65.2	74.0	124.4*	61.4	121.7*	99.9	144.9*	71.7
	GABA(A)	104.8	92.1	288.9*	71.4	50.8	73.0	34.9*	73.0	33.3*	42.9
	NMDA	24.9*	71.7	107.0	101.7	51.5*	93.8	74.0*	67.7*	60.9*	42.9*
	mACh	93.2	61.5	109.5	131.6	142.4*	131.5	126.2	89.5	110.8	81.6
neurochemical enzymes	MAO	72.3*	103.7	53.3*	98.8	52.1*	96.7	85.6*	99.6	63.5*	100.1
	GAD	81.6	80.6	91.4	89.0	73.6	86.5	92.1	101.7	93.6	88.5
	GABA-T	120.9*	66.6*	168.6*	158.1*	150.5*	108.3	123.7	112.7	67.1*	80.3
	AChE	53.4*	100.4	68.0*	95.6	64.0*	79.3*	62.3*	99.6	50.2*	93.9

(Table 3). However, these results should be interpreted with caution because the [3H]-muscimol binding assay (used to label GABA(A) binding sites) suffers from high background noise (nonspecific binding was ~59% in the presence of 100 μM unlabeled muscimol) and poor intrawell variability (~22% variation). Alternative assays to label this receptor are currently being sought. Nevertheless, a single extract (nonpolar, hexane derived) from the primary effluent significantly increased GABA(A) receptor binding by nearly 3-fold (Table 3). In contrast, a water-soluble fraction obtained from the secondary effluent, using both solvent series and PVPP extraction methods significantly inhibited ligand binding by ~65−67% (Table 3). The other extracts derived from the secondary effluent also reduced receptor binding, but not to a level of statistical significance.

Synaptic levels of GABA are tightly regulated by two metabolic enzymes: GAD and GABA-T. The function of GAD, a rate-limiting enzyme, is to catalyze the decarboxylation of glutamate into GABA (*40*). Here we found that none of the extracts significantly affected the activity of GAD (Table 3). The regulation of GABA is also served by the catabolic enzyme GABA-T. Pharmacological inhibition of GABA-T using the antiepileptic drug, vigabatrin (γ-vinyl-GABA), increases hypothalamic GABA levels and promotes LH release in goldfish (*18*). Here we found two of the water-soluble extracts (from both primary and secondary effluent) significantly reduced GABA-T activity by ~40%. However, several extracts from the primary effluent derived using both solvent series and PVPP extraction methods significantly increased GABA-T activity by 21−69% (Table 3). While it is not possible to draw any firm conclusions based on the GAD, GABA-T, and GABA(A) results (as the results vary across effluent type, extraction solvent, and extraction method), the presence of GABAergic modulating compounds in effluents are not surprising as a recent in vitro experiment showed that several botanical extracts can affect the enzymatic activities of both GAD and GABA-T in rat brains (*30*). Perturbation of GABA levels in fish would be predicted to disrupt the fine control of LH release, contributing to the antireproductive effects of effluents.

The NMDA receptor, the major glutamatergic ionotropic receptor, is present in the fish hypothalamus (*41*) and GnRH brain centers (*42*). Similar to the GABAergic pathway, agonist stimulation of the NMDA receptor promotes LH release (*19, 43*). Here we found that several extracts significantly inhibited [3H]-MK801 binding to the NMDA receptor but the results were not consistent among effluent type, extraction method, and extraction solvent (Figure 3). Whole animal studies have clearly shown that blockage of the NMDA receptor can adversely affect reproduction. Injection of platyfish (*Xiphophorus maculates*) with the NMDA receptor antagonist, MK-801, delayed the onset of puberty (*21*). Rodent studies have shown NMDA receptor antagonists can disrupt LH release and impair reproductive behavior in mammalian models (*20*). Owing to the ubiquity of NMDA receptors in fish brain and the involvement of glutamate in several neuronal processes, disruptions to this pathway represents a plausible mechanism by which effluents may disrupt reproduction in wild fish.

The cholinergic system has important roles in neurodevelopment and reproductive behavior (*44*). Here we found that extracts from pulp and paper mill effluent affected mACh receptor binding and AChE activity. For the mACh receptor, ligand binding was only affected by one extract (ethanol fraction derived from PVPP extraction of primary effluent significantly increased binding). Several extracts inhibited AChE enzyme activity (Table 3). Interestingly, water-derived extracts of secondary effluent inhibited AChE activity while in the primary effluent, nonwater extracts caused enzyme inhibition. Inhibition of AChE is widely used as a biomarker of organophosphate pesticide exposure in birds, fish, and wildlife (*45*), and inhibitions of enzyme activity, in vivo, to the same extent measured here (20−50%) are associated with mild symptoms of clinical poisoning, such as headaches and fatigue (*46*).

Chemical and thermo-mechanical processing of wood results in complex mixtures of compounds, many of which are not yet identified (*1*). Some known compounds are biologically active phytochemicals, notably polyphenolics and terpenes. Previous experiments have established that some of these phytochemicals are neuroactive (*30, 32, 33*). Here, our work shows that effluent extracts from a pulp and paper mill are also neuroactive in vitro. An interesting observation was made by comparing the in vitro responses between the primary and secondary effluents (Table 3). In the primary effluent subjected to the solvent series extraction, the greatest effects were generally found in extracts derived from the organic, lipophilic solvents (EtOAc and hexane). These lipophilic extracts are likely rich in terpenes which have recently been associated with reductions of sex steroid levels in fish (*47*). In the PVPP extraction of primary effluent the EtOH fraction (phenolic fraction) was clearly more active than the water fractions as 6 of 8 neurochemical parameters were significantly affected (versus 1 for water). In contrast, in the secondary effluents, the greatest neuroactive responses were found in the water (11 of 16 possible neurochemical parameters were significantly affected) extracts derived from both solvent series and PVPP extraction methods. These observations suggest that more polar, water soluble compounds can affect brain neurochemistry following biotreatment. The role of biotreatment systems on how mill effluents affect fish reproduction is the subject of much debate, with

conflicting reports of effluents after treatment showing improvements, no difference, or an exacerbation of effects (*5*).

In conclusion, this work unequivocally shows that components within pulp and paper mill effluent can interact with, and disrupt, key neurochemical receptors and enzymes important to fish reproduction. These results suggest a novel and plausible mechanism by which mill effluents impact fish reproduction. To establish causal linkages, future experiments should determine if the aforementioned receptors and enzymes are affected in fish exposed to mill effluents in the laboratory and in receiving environments. Isolation and identification of neuroactive substances in the mill effluent will also be required to fully understand how effluent-induced perturbations to neuroendocrine pathways result in reproductive effects. Owing to the heterogeneous composition of effluents and their dynamic nature, further in vitro studies should be conducted on other effluents to see if similar neurochemical effects are elicited. If so, incorporating neurochemical end points in an evaluation of the efficacy of biotreatment systems at different mills may indicate which systems and operating conditions are effective at removing substances affecting fish reproduction.

Acknowledgments

We thank Tibor Kovacs and Pierre Martel for supplying test effluents and providing insightful comments into the preparation of this paper. This work was funded by the NSERC Strategic grants program (V.L.T. and J.T.A.). No conflict of interest is declared, and the authors assume sole responsibility for the contents of this manuscript.

Literature Cited

(1) Hewitt, L. M.; Parrott, J. L.; McMaster, M. E. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: sources and characteristics of bioactive substances. *J. Toxicol. Environ. Health, B* **2006**, *9*, 341–356.

(2) McMaster, M. E.; Hewitt, L. M.; Parrott, J. L. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: field studies and mechanistic research. *J. Toxicol. Environ. Health, B* **2006**, *9*, 319–339.

(3) Parrott, J. L.; McMaster, M. E.; Hewitt, L. M. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: development and application of fish bioassays. *J. Toxicol. Environ. Health, B* **2006**, *9*, 297–317.

(4) van den Heuvel, M. R. Overview of fish health studies—Progress since 2000. In *Pulp & Paper Mill Effluent Environmental Fate & Effects*; Borton, D. L., Hall, T. J., Fisher, R. P., Thomas, J. F., Eds. DesTech Publications, Inc: Lancaster, PA, 2004; pp 3−13.

(5) Hewitt, L. M.; Kovacs, T. G.; Dube, M. G.; MacLatchy, D. L.; Martel, P. H.; McMaster, M. E.; Paice, M. G.; Parrott, J. L.; van den Heuvel, M. R.; van der Kraak, G. J. Altered reproduction in fish exposed to pulp and paper mill effluents: roles of individual compounds and mill operating conditions. *Environ. Toxicol. Chem.* **2008**, *27*, 682–697.

(6) Van Der Kraak, G.; Munkittrick, K. R.; M.E., M.; D.M., M. A comparison of bleached kraft pulp mill effluent, 17b-estradiol and b-sitosterol effects on reproductive function in fish. In *Principles and Processes for Evaluating Endocrine Disruption in Wildlife*; Kendall, R. J.; Dickerson, R. L.; Suk, W. A.; Giesy, J. P., Eds.; SETAC Press: Pensacola, Fl, 1998; pp 249−265.

(7) Tremblay, L.; Van Der Kraak, G. Use of a series of homologous in vitro and in vivo assays to evaluate the endocrine modulating actions of β-sitosterol in rainbow trout. *Aquat. Toxicol.* **1998**, *43*, 149–162.

(8) Ellis, R. J.; van den Heuvel, M. R.; Bandelj, E.; Smith, M. A.; McCarthy, L. H.; Stuthridge, T. R.; Dietrich, D. R. In vivo and in vitro assessment of the androgenic potential of a pulp and paper mill effluent. *Environ. Toxicol. Chem.* **2003**, *22*, 1448–1456.

(9) Larsson, D. G. J.; Hallman, H.; Förlin, L. More male fish embryos near a pulp mill. *Environ. Toxicol. Chem.* **2000**, *19*, 2911–2917.

(10) Parks, L. G.; Lambright, C. S.; Orlando, E. F.; Guillette, L. J., Jr.; Ankley, G. T.; Gray, L. E., Jr. Masculinization of female mosquitofish in kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicol. Sci.* **2001**, *62*, 257–267.

(11) Trudeau, V. L.; Spanswick, D.; Fraser, E. J.; Lariviere, K.; Crump, D.; Chiu, S.; MacMillan, M.; Schulz, R. W. The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. *Biochem. Cell Biol.* **2000**, *78*, 241–259.

(12) Van Der Kraak, G.; Zacharewski, T.; Janz, D. M.; Sanders, B. M.; Gooch, J. W. Comparative endocrinology and mechanisms of endocrine modulation in fish and wildlife. In *Principles and Processes for Evaluating Endocrine Disruption in Wildlife*; Kendall, R. J.; Dickerson, R. L.; Suk, W. A.; Giesy, J. P., Eds.; SETAC Press: Pensacola, FL, 1998; pp 97−119.

(13) Peter, R. E.; Chang, J. P.; Nahorniak, C. S.; Omeljaniuk, R. J.; Sokolowska, M.; Shih, S. H.; Billard, R. Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. *Recent Prog. Horm. Res.* **1986**, *42*, 513–548.

(14) Habibi, H. R.; Marchant, T. A.; Nahorniak, C. S.; Van der Loo, H.; Peter, R. E.; Rivier, J. E.; Vale, W. W. Functional relationship between receptor binding and biological activity for analogs of mammalian and salmon gonadotropin-releasing hormones in the pituitary of goldfish (*Carassius auratus*). *Biol. Reprod.* **1989**, *40*, 1152–1161.

(15) Chang, J. P.; Yu, K. L.; Wong, A. O.; Peter, R. E. Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release in vitro in goldfish. *Neuroendocrinology* **1990**, *51*, 664–674.

(16) Kah, O.; Trudeau, V. L.; Sloley, B. D.; Chang, J. P.; Dubourg, P.; Yu, K. L.; Peter, R. E. Influence of GABA on gonadotrophin release in the goldfish. *Neuroendocrinology* **1992**, *55*, 396–404.

(17) Martyniuk, C. J.; Chang, J. P.; Trudeau, V. L. The effects of GABA agonists on glutamic acid decarboxylase, GABA-transaminase, activin, salmon gonadotrophin-releasing hormone and tyrosine hydroxylase mRNA in the goldfish (*Carassius auratus*) neuroendocrine brain. *J. Neuroendocrinol.* **2007**, *19*, 390–396.

(18) Trudeau, V. L.; Sloley, B. D.; Peter, R. E. GABA stimulation of gonadotropin-II release in goldfish: involvement of GABAA receptors, dopamine, and sex steroids. *Am. J. Physiol.* **1993**, *265*, R348–R355.

(19) Trudeau, V. L.; Sloley, B. D.; Peter, R. E. Testosterone enhances GABA and taurine but not *N*-methyl-D,L-aspartate stimulation of gonadotropin secretion in the goldfish: possible sex steroid feedback mechanisms. *J. Neuroendocrinol.* **1993**, *5*, 129 136.

(20) Mahesh, V. B.; Brann, D. W. Regulatory role of excitatory amino acids in reproduction. *Endocrine Rev.* **2005**, *28*, 271–280.

(21) Flynn, K. M.; Miller, S. A.; Sower, S. A.; Schreibman, M. P. Sexually dimorphic effects of NMDA receptor antagonism on brain-pituitary-gonad axis development in the platyfish. *Comp. Biochem. Physiol. C* **2002**, *131*, 9–18.

(22) Krsmanovic, L. Z.; Mores, N.; Navarro, C. E.; Saeed, S. A.; Arora, K. K.; Catt, K. J. Muscarinic regulation of intracellular signaling and neurosecretion in gonadotropin-releasing hormone neurons. *Endocrinology* **1998**, *139*, 4037–4043.

(23) Popesku, J T.; Martyniuk, C. J.; Mennigen, J.; Xiong, H.; Zhang, D.; Xia, C.; Cossins, A. R.; Trudeau, V. L. The goldfish (*Carassius auratus*) as a model for neuroendocrine signaling. *Mol. Cell. Endocrinol.* **2008**, *293*, 43–56.

(24) Pisha, E.; Chai, H.; Lee, I.-S.; Chagwedera, T. E.; Farnsworth, N. R.; Cordell, G. A.; Beecher, C. W. W.; Fong, H. H. S.; Kinghorn, A. D.; Brown, D. M.; Wani, M. C.; Wall, M. E.; Hieken, T. J.; Das Gupta, T. K.; Pezzuto, J. M. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nat. Med.* **1995**, *1*, 1046–1051.

(25) Charmont, S.; Jamet, E.; Pont-Lezica, R.; Canut, H. Proteomic analysis of secreted proteins from *Arabidopsis thaliana* seedlings: improved recovery following removal of phenolic compounds. *Phytochemistry* **2005**, *66*, 453–461.

(26) Basu, N.; Scheuhammer, A. M.; Sonne, C.; Letcher, R. J.; Born, E. W.; Dietz, R. Is dietary mercury of neurotoxicological concern to wild polar bears (*Ursus maritimus*)?*Environ. Toxicol. Chem.* **2009**, *28*, 133–140

(27) Basu, N.; Scheuhammer, A.; Grochowina, N.; Klenavic, K.; Evans, D.; O'Brien, M.; Chan, H. M. Effects of mercury on neurochemical receptors in wild river otters (*Lontra canadensis*). *Environ. Sci. Technol.* **2005**, *39*, 3585–3591.

(28) Basu, N.; Klenavic, K.; Gamberg, M.; O'Brien, M.; Evans, D.; Scheuhammer, A. M.; Chan, H. M. Effects of mercury on neurochemical receptor-binding characteristics in wild mink. *Environ. Toxicol. Chem.* **2005**, *24*, 1444–1450.

(29) Basu, N.; Scheuhammer, A. M.; Evans, R. D.; O'Brien, M.; Chan, H. M. Cholinesterase and monoamine oxidase activity in relation to mercury levels in the cerebral cortex of wild river otters. *Hum. Exp. Toxicol.* **2007**, *26*, 213–220.
(30) Awad, R. L.; Levac, D.; Cybulska, P.; Merali, Z.; Trudeau, V. L.; Arnason, J. T. Effects of traditionally used anxiolytic botanicals on enzymes of the γ-aminobutyric acid (GABA) system. *Can. J. Physiol. Pharmacol.* **2007**, *85*, 933–942.
(31) Vacher, C.; Mananos, E. L.; Breton, B.; Marmignon, M. H.; Saligaut, C. Modulation of pituitary dopamine D1 or D2 receptors and secretion of follicle stimulating hormone and luteinizing hormone during the annual reproductive cycle of female rainbow trout. *J. Neuroendocrinol.* **2000**, *12*, 1219–1226.
(32) Viana, A.; do Rego, J.-C.; von Poser, G.; Ferraz, A.; Heckler, A. P.; Costentin, J.; Kuze Rates, S. M. The antidepressant-like effect of *Hypericum caprifoliatum* Cham & Schlecht (Guttiferae) on forced swimming test results from an inhibition of neuronal monoamine uptake. *Neuropharmacology* **2005**, *49*, 1042–1052.
(33) Houghton, P. J.; Howes, M. J. Natural products and derivatives affecting neurotransmission relevant to Alzheimer's and Parkinson's disease. *Neurosignals* **2005**, *14*, 6–22.
(34) Dube, M. G.; MacLatchy, D. L. Endocrine responses of *Fundulus heteroclitus* to effluent from a bleached-kraft pulp mill before and after installation of reverse osmosis treatment of a waste stream. *Environ. Toxicol. Chem.* **2000**, *19*, 2788–2796.
(35) Kovacs, T. G.; Martel, P.; Ricci, M. A shortened adult fathead minnow reproduction test developed for investigation of cause and investigation of solution work involving pulp and paper mill effluents. *Water Qual. Res. J. Can.* **2007**, *42*, 21–100.
(36) Sloley, B. D.; Trudeau, V. L.; Peter, R. E. Dopamine catabolism in goldfish (*Carassius auratus*) brain and pituitary: Lack of influence of catecholestrogens on dopamine catabolism and gonadotropin secretion. *J. Exp. Zool.* **1992**, *263*, 398–405.
(37) Khan, I. A.; Thomas, P. Aroclor 1254-induced alterations in hypothalamic monoamine metabolism in the Atlantic croaker (*Micropogonias undulatas*): correlation with pituitary gonadotropin release. *Neurotoxicology* **1997**, *18*, 553–560.
(38) Szczerbik, P.; Mikolajczyk, T.; Sokolowska-Mikolajczyk, M.; Socha, M.; Chyb, J.; Epler, P. Influence of long-term exposure to dietary cadmium on growth, maturation and reproduction of goldfish (subspecies: Prussian carp *Carassius auratus gibelio B.*). *Aquat. Toxicol.* **2006**, *77*, 126–135.
(39) Trudeau, V. L. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Carassius auratus*. *Rev. Reprod.* **1997**, *2*, 55–68.
(40) Martin, D. L.; Rimvall, K. Regulation of gamma-aminobutyric acid synthesis in the brain. *J. Neurochem.* **1993**, *60*, 395–407.
(41) Bottai, D.; Dunn, R. J.; Ellis, W.; Maler, L. N-methyl-D-aspartate receptor 1 mRNA distribution in the central nervous system of the weakly electric fish *Apteronotus leptorhynchus*. *J. Comp. Neurol.* **1997**, *389*, 65–80.
(42) Flynn, K. M.; Schreibman, M. P.; Magliulo-Cepriano, L. Developmental changes in NMDA receptor expression in the platyfish brain. *Brain Res.* **1997**, *771*, 142–146.
(43) Flett, P. A.; Van Der Kraak, G.; Leatherland, J. F. Effects of excitatory amino acids on in vivo and in vitro gonaodotropin and growth hormone secretion in testosterone-primed immature rainbow trout *Onchorhynchus mykiss*. *J. Exp. Zool.* **1994**, *268*, 390–399.
(44) Slotkin, T. A. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. *Toxicol. Appl. Pharmacol.* **2004**, *198*, 132–151.
(45) Mineau, P. *Cholinesterase-Inhibiting Insecticides*; Elsevier: New York, 1991.
(46) McQueen, M. J. Clinical and analytical considerations in the utilization of cholinesterase measurements. *Clin. Chim. Acta* **1995**, *237*, 91–105.
(47) Belknap, A. M.; Solomon, K. R.; MacLatchy, D. L.; Dube, M. G.; Hewitt, L. M. Identification of compounds associated with testosterone depressions in fish exposed to bleached kraft pulp and paper mill chemical recovery condensates. *Environ. Toxicol. Chem.* **2006**, *25*, 2322–2333.

ES802552M

Appendix 3

Environmental Science & Technology

Article

pubs.acs.org/est

Evaluating the Potential of Effluents and Wood Feedstocks from Pulp and Paper Mills in Brazil, Canada, and New Zealand to Affect Fish Reproduction: Chemical Profiling and In Vitro Assessments

Craig B. Milestone,*[,†,‡] Rodrigo Orrego,[§] Philip D. Scott,[∥] Andrew Waye,[⊥] Jagmohan Kohli,[‡] Brian I. O'Connor,[#] Brendan Smith,[¶] Heidi Engelhardt,[¶] Mark R. Servos,[¶] Deborah L. MacLatchy,[†] D. Scott Smith,[∥] Vance L. Trudeau,[⊥] John T. Arnason,[⊥] Tibor Kovacs,[#] Tatiana Heid Furley,[▽] Alison H. Slade,[◆] Douglas A. Holdway,[§] and L. Mark Hewitt[‡]

[†]Department of Biology, Wilfrid Laurier University, Waterloo, ON, N2L 3C5, Canada

[‡]National Water Research Institute, Environment Canada, Burlington, ON, L7R 4A6, Canada

[§]Faculty of Science, University of Ontario Institute of Technology, Oshawa, ON, L1H 7K4, Canada

[∥]Department Chemistry, Wilfrid Laurier University, Waterloo, ON, N2L 3C5, Canada

[⊥]Department of Biology, University of Ottawa, Ottawa, ON, K1N 6N5, Canada

[#]FPInnovations, Pointe-Clare, QC, H9R 3J9, Canada

[¶]Department of Biology, University of Waterloo, Waterloo, ON, N2L 3G1, Canada

[▽]Aplysia Institute, Vitória, ES, 29090-210, Brazil

[◆]Scion, Rotorua, New Zealand

Supporting Information

ABSTRACT: This study investigates factors affecting reproduction in fish exposed to pulp and paper mill effluents by comparing effluents from countries with varying levels of documented effects. To explore the hypothesis of wood as a common source of endocrine disrupting compounds, feedstocks from each country were analyzed. Analyses included in vitro assays for androgenic activity (binding to goldfish testis androgen receptors), estrogenic activity (yeast estrogen screen), and neurotransmitter enzyme inhibition (monoamine oxidase and glutamic acid decarboxylase). Chemical analyses included conventional extractives, known androgens, and gas chromatograph index (GCI) profiles. All effluents and wood contained androgenic activity, particularly in nonpolar fractions, although known androgens were undetected. Effluents with low suspended solids, having undergone conventional biotreatment had lower androgenic activities. Estrogenic activity was only associated with Brazilian effluents and undetected in wood. All effluents and wood inhibited neurotransmitter enzymes, predominantly in polar fractions. Kraft elemental chlorine free mills were associated with the greatest neurotransmitter inhibition. Effluent and wood GCI profiles were correlated with androgenic activity and neurotransmitter enzyme inhibition. Differences in feedstock bioactivities were not reflected in effluents, implying mill factors mitigate bioactive wood components. No differences in bioactivities could be discerned on the basis of country of origin, thus we predict effluents in regions lacking monitoring would affect fish reproduction and therefore recommend implementing such programs.

INTRODUCTION

While many major environmental issues regarding pulp and paper effluent discharges, such as biochemical oxygen demand, suspended solids and nutrients management, and dioxins and furan releases, have largely been addressed, one major issue remains: the effects of mill effluents on fish reproduction. Documented reproductive effects have included delayed sexual maturity, decreased gonad size, reduced gonadal and circulating sex steroids, and reduced secondary sex characteristics in wild fish. Initially reported in Scandinavia,[1] effects have been reported throughout pulp producing nations, including Canada, the U.S.A., and New Zealand (reviewed in refs 2−4). Laboratory and in situ studies report a suite of effects with the most consistent related to androgenic and estrogenic modes of action.[5−7] The rapid onset of some effects (e.g., sex steroids

Received: September 26, 2011
Revised: December 18, 2011
Accepted: December 23, 2011
Published: December 23, 2011

in wild fish and egg production in laboratory fish), and their reversal following exposure cessation,[8−10] suggest that causative compounds for these responses are readily bioavailable and metabolized quickly. Evidence of these effects exists from both regulatory programs, such as the Canadian Environmental Effect Monitoring (EEM) Program,[11] and extensive research studies, such as in New Zealand.[3]

Despite the evidence, a relationship between mill process parameters, effluent treatment type and specific reproductive effects in fish remains unclear.[2] It has been hypothesized that a common origin of causative agents exists, including the wood furnish or additives. It is thought that these compounds survive or are possibly altered during pulping, bleaching, and effluent treatment.[2]

In Canada and New Zealand, the pulp and paper industry is well established, where mills have been retrofitted to address environmental concerns as they arose. Upgrades have included the implementation of elemental chlorine free (ECF) bleaching and biological treatment. Continual changes in both production and wastewater treatment have been shown to be beneficial for minimizing effluent-related effects, as evidenced at one mill in New Zealand.[12] The newest mills in the world are currently located in South America and have been constructed with all these latest process modifications included; however, there is little information regarding effluent effects. South American pulp-producing countries have not yet been mandated to conduct monitoring, and as such, the only available information is generated from recent research. This research shows that effluent from state-of-the-art mills in Chile can cause estrogenic effects in laboratory,[13,14] caged,[15] and wild fish.[16] This lack of information hinders the ability of mature pulp and paper sectors in other countries from implementing effective solution-oriented changes. Historically, comparisons between studies, and especially between different pulp producing nations, have been difficult. This is due to the varied approaches involving different fish species, experimental conditions, responses evaluated and study objectives.[17] A comprehensive evaluation of effluents using the same protocols and end points is therefore needed to address this knowledge gap.

New tools and approaches developed as part of a Canadian national initiative are offering leads toward minimizing effects on fish reproduction and the ability to compare mill effluents more consistently. These tools include a gas chromatographic index (GCI), which provides a measure of extractable organics, and for kraft mill effluents, correlates positively to BOD, correlates negatively to egg production in fathead minnows,[18] and is linked to effects in wild fish.[19] New evidence of impacts in the hypothalamus provides a plausible mechanism where rapidly manifested effects may be initiated through interactions with receptors and enzymes involved in neurotransmitter synthesis or metabolism.[20−22] The hypothalamus−pituitary−gonad reproductive axis is tightly regulated by the neurotransmitters gamma-aminobutyric acid (GABA) and dopamine in many teleost species.[23−25] Dopamine is a potent inhibitor of gonadotropin-releasing hormone (GnRH) in the brain and luteinizing hormone (LH) in the pituitary, thereby controlling a multitude of neurotransmitters and sex steroids. Conversely, GABA stimulates GnRH and blocks dopamine's inhibition of LH to cause LH release. Monoamine oxidase (MAO) catabolizes dopamine, while glutamic acid decarboxylase (GAD) catalyzes the decarboxylation of glutamate into GABA. Inhibition of these enzymes would lead to either increased levels of dopamine or decreased levels of GABA in the brain, either of which would be inhibitory to fish reproduction. Hypothalamic effects have been linked to spawning inhibition with a limited number of Canadian mill effluents.[22]

The objective of this study is to evaluate effluents from countries where reproductive effects and industries are well established (Canada, New Zealand), to countries where mills are state-of-the-art, with no effects data (Brazil). To explore the hypothesis of wood extractives as a source of endocrine disrupting compounds in final effluents, representative samples of wood feedstocks from each country were incorporated. Final mill effluents and feedstocks were evaluated for (i) androgenic and estrogenic activities, (ii) neuroactive substances affecting the neuroendocrine control of ovulation, and (iii) conventional effluent extractives, known androgens and GCI profiles to determine if any relationships exist between bioassay responses, feedstock and effluent chemistry, and mill operational parameters. Selected effluent extracts are being separately evaluated for their effect on reproduction in rainbow trout.

EXPERIMENTAL SECTION

All solvents were HPLC grade unless otherwise specified (Optima grade, Fisher, Ottawa, ON, Canada), gases were ultrahigh purity grade (Air Liquide, Hamilton, ON, Canada).

Mill Selection. Final effluents and wood feedstocks were collected from four Canadian mills, five Brazilian mills, and two mills from New Zealand representative of the pulping and bleaching processes, effluent treatment systems, and wood types used globally (Table 1). In addition to conventional mill metrics (biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), flow), information regarding other parameters (wood type switching, nonmill wastewaters, tertiary treatment) were obtained through a comprehensive questionnaire. To avoid any changes to effluent quality that may occur during handling and international shipment, all extractions were conducted within the country of origin using supplies and a protocol distributed by the host Canadian laboratory. The stability of extracted components on frozen SPE cartridges was verified prior to commencement of the study (Figure S1, Supporting Information).

Effluent Collections. Extraction and filtration supplies were sent to participating laboratories in Brazil (Aplysia, Vitória, ES) and New Zealand (Scion, Rotorua). Samples (6 L) of final treated effluent from each mill were collected as grab or 24-h composites in prewashed 1 L glass bottles. Extractions were performed on the same day (New Zealand), or within two days (stored at 4 °C, Brazil, Canada).

Effluent Extractions. Extraction methods were based on the approach of Orrego et al.[26] and modified by including extractions of filtered solids, use of a less selective SPE solid phase and separation of compounds by polarity. Room temperature effluent pH (∼7−8) was lowered using hydrochloric acid (3.0 M) to pH 4.0 (±0.2) and filtered through glass fiber filters (45 mm, 1.2 μm, Whatman GF-C, VWR, Mississauga, ON, Canada). Fouled filters were air-dried, wrapped in hexane-rinsed aluminum foil and sealed in polyethylene bags for shipment to Canada. To the pH-adjusted final effluent filtrate, 2% (v/v) methanol was added and eluted (500 mL) through solid phase extraction (SPE) cartridges (1 g, Oasis HLB, Waters, Mississauga, ON, Canada) preconditioned with 2 × 20 mL of dichloromethane, methanol, and water (pH = 4.0). Cartridges were washed with 10 mL water (pH = 4.0) and dried for 1 h. Loaded SPE cartridges and fouled filter

Table 1. Participating Mills Sampled in Canada, Brazil, and New Zealand[a]

										treatment efficiency			
		mill type	ECF bleaching sequence	wood type at sampling	wood type switch	storm water	other wastewater	treatment type	wastewater flow (m^3/ADMT)	% BOD removal	% COD removal	TSS % removal	tertiary
Canada	Mill A	ECF kraft	$DE_{op}D$	hardwood (aspen)	yes	yes	no	AS	44.5	98	74.3	94.1	no
	Mill B	TMP	na[b]	softwood mix (75% spruce/25% pine)	no	no	no	AS	45.9	99	93	91	no
	Mill C	TMP	na	softwood mix (spruce 85%/15% balsam fir)	no	small	yes[c]	ASB	30.6	74.6	np[h]	np	no
	Mill D	ECF kraft	$DE_{op}DED$	softwood mix	yes	no	no	novel[g]	23.8	92	41	np	no
Brazil	Mill A	ECF kraft	$D_oE_{op}DD$ D_oE_oPPP	hardwood (eucalypt)	no	yes	yes	ASB	28.8	89.9	62.5	75	no
	Mill B	kraft	unbleached	softwood mix (*Pinus taeda* and *elliotos*)	no	no	no	AS	52.4	93.8	88	93.2	no
	Mill C	ECF kraft	$D_{hot}O_pD$	softwood and hardwood (*Pinus radiata* and eucalyptus)	no	no	no	AS	44.1	94	80	np	yes[I]
	Mill D	ECF kraft	$OADE_{op}D$	hardwood (eucalyptus)	no	no	no	AS	30.6	>99	88.3	95	np
	Mill E	ECF kraft	$D_{hot}E_{op}DP$	hardwood (eucalyptus)	no	no	yes[d]	AS	21.8	97.2	81	86	np
New Zealand	Mill A	ECF kraft	$OODE_{op}D$	softwood (95%) and hardwood (5%) (*Pinus radiata* and eucalyptus)	no	yes	yes[e]	ASB	53.2	92.3	np	76	no
	Mill B	ECF kraft (49%) and TMP (51%)	D(EOP)PD and (EOP)(P)	kraft, hardwood (eucalyptus); TMP, softwood (*Pinus radiata*)	yes (kraft); no (TMP)	yes	yes[f]	ASB	74.7	89.7	np	np	no

[a]ADMT = air dried metric tonne, ECF-*K* = elemental chlorine free (ClO_2) bleached kraft pulp (*D* = chlorine dioxide, *E* = extraction stage, $_{op}$ = with oxygen and hydrogen peroxide, $_p$ = with hydrogen peroxide, $_o$ = with oxygen, *A* = acid hydrolysis), U−*K* = Unbleached kraft, TMP = Thermo-mechanical pulping, AS = activated sludge secondary treatment system, ASB = activated stabilisation basin (aerated lagoon) treatment system, BOD = biological oxygen demand, COD = chemical oxygen demand, TSS =total suspended solids [b]Not applicable. [c]Small volume from nearby oil refinery. [d]Sanitary sewer enters in to treatment system. [e]Small volume of underflow from adjacent landfills. [f]Whitewater from adjacent tissue mill, and leachate from adjacent landfill and from natural wetland system. [g]Novel treatment system: non-ASB or -AS. [h]Data not provided by mill. [I]Mill uses ultrafiltration on 40% of flow prior to discharge; sample for study was taken before tertiary treatment.

dx.doi.org/10.1021/es203382c | Environ. Sci. Technol. 2012, 46, 1849–1858

papers were shipped frozen to be eluted and extracted in Burlington, Canada, ON, alongside Canadian mill effluents shipped directly. Spiking experiments with phenolics, diterpenes, and sterols showed that frozen storage of loaded SPE cartridges up to two weeks produced adequate recoveries (Figure S1, Supporting Information).

Fouled filter papers were Soxhlet extracted for 18 h sequentially with dichloromethane and methanol producing nonpolar (**FP-DCM**) and polar (**FP-MeOH**) fractions. Loaded SPE cartridges were eluted sequentially using 40 mL solvent to produce two fractions: dichloromethane (**SPE-DCM**) and methanol (**SPE-MeOH**). All DCM fractions were dried using anhydrous Na_2SO_4, concentrated using rotary evaporation and evaporated under nitrogen to just-dryness and reconstituted for chemical (toluene) and biological (methanol) assessments at 1 L final effluent/mL solvent.

Wood Feedstock Extractions. Chips were sampled directly from mill stockpiles and shipped with effluents (Canadian mills) or loaded filters and SPE cartridges (Brazilian, New Zealand mills). Chips were air-dried and milled to ~1 cm^3 pieces, and Soxhlet extracted (24 h, 1 L) sequentially with hexane, dichloromethane, and acetone to ensure a best possible discrimination of bioactive compounds by polarity. After each solvent, chips were air-dried. Extracts were dried and concentrated as described above to 2.5 g/mL equivalents.

Gas Chromatography-Tandem Mass Spectrometry. All fractions were methylated using freshly prepared diazomethane (Diazald, Sigma Aldrich, Mississauga ON, Canada) in diethyl ether. Gas chromatography-tandem mass spectrometry (GC-MS-MS; Agilent 7890 GC, 7000B triple-quadrupole MS) analyses were performed as follows: HP-MS5 column (Agilent, 30 m × 0.25 mm, 0.25 μm), programmed 90 °C for 0.5 min, 5 °C/min to 300 °C for 10 min; splitless injection temperature 270 °C, helium carrier (1.3 mL/min). Positive ion electron impact full scan mass spectra (unit resolution, 50−500 m/z) were used to generate total ion chromatograms (TICs). Prior to injection, an internal standard (C19 alkane, Restek, Bellefont, PA, USA) was added to account for matrix effects. The GCIs were calculated by integrating the total peak areas (1.7−10 min) and adjusting to the response of the internal standard (area of C19 molecular ion m/z = 178; average relative response 0.96 ± 0.08; n = 43). Adjusted peak areas were then normalized against corresponding method blanks, each of which were assigned a GCI = 1.0 (modified from ref 8).

Effluent chemicals functioning as androgens (progesterone, androstenediene (AD), androstadienedione (ADD),[27−29] and manool[30]) were analyzed using the same GC conditions, using multiple reaction monitoring mode: progesterone (quantifier 313/191 and qualifier 313/299), AD (quantifier 285/244 and qualifier 285/124), ADD (quantifier 123/107 and qualifier 123/79), and manool (quantifier 272/257 and qualifier 272/135). Method detection limits were: progesterone (0.655 μg/L and 0.275 μg/g), AD (0.406 μg/L and 0.163 μg/g), ADD (5.5 μg/L and 2.2 μg/g) for effluent and wood extracts respectively, and quantifications were based on 6-point calibrations. Detection limits for phenolics, diterpenes, and sterols (Figure S1, Supporting Information) are reported elsewhere.[30] Resin acids (eight pimaric, sandaracopimaric, isopimaric, palustric, levopimaric, dehydroabietic, and abietic acids) and fatty acids (two linoleic and oleic acids) were quantified as previously described.[31]

Androgen Receptor Binding Assay. Androgen receptor (AR) ligands in fractions of wood and effluents were evaluated using a competitive binding assay with goldfish testis androgen receptors.[32] Briefly, androgen receptors were isolated from goldfish (*Carassius auratus*, 50−100 g) testes (2 pools from each of 7 males; 8 g total). Scintillation tubes contained 1% (v/v) methanol vehicle; previously shown to not affect results.[32] Dilutions were performed as required, to ensure specific binding and to obtain testosterone equivalents (TEq) from the linear portion of the standard curve. Each fraction was incubated in triplicate using both AR preparations. Method detection limits were 128 ng/L TEq effluent and 51 ng/g TEq wood.

Yeast Estrogen Screening Assay. Fractions of pulp mill effluents and feedstocks were evaluated for estrogen agonists using a yeast (*Saccharomyces cerevisiae*) estrogen screen (YES).[33] Results are represented as 17β-estradiol equivalents, with a method detection limit of 10.4 ng/L and 4.1 ng/g for effluent and wood, respectively.

Neurotransmitter Enzyme Assays. Effluent and wood fractions were evaluated for substances potentially affecting the neuroendocrine control of ovulation through the inhibition of MAO and GAD. For the MAO assay, whole goldfish brains were sonicated immediately after dissection in cold Na/K buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4) with 0.5% (v/v) Triton X-100. Sonicated tissue was centrifuged at 15 000 × g at 4 °C for 10 min, supernatants removed and stored (−80 °C). For the GAD assay, brains were homogenized in 1:10 (w/v) 10 mM phosphate buffer (pH 7.5) and stored (−80 °C).

MAO activity was measured according to Basu et al.,[20,34] with modifications, using the Amplex Red Monoamine Oxidase Assay Kit (Invitrogen, Burlington, ON, Canada). Fractions were preincubated with tissue (n = 4) for 30 min before reaction initiation at a final concentration of 2.5 μg/mL. The MAO inhibitor clorgyline (1 μM) was used as a positive control for MAO inhibition while 10 μM H_2O_2 was used as a positive control for resorufin production (λ_{ex} = 544 nm, λ_{em} = 590 nm) for 2 h, following reaction initiation using a SpectroMax M5 (Moleculare Devices, Sunnyvale, CA, U.S.A.). For background values, no protein was added to wells.

GAD activity was measured in triplicate by incubating 50 μg brain homogenate with fractions (25 μg/mL) in 10 mM phosphate buffer (60 μM pyridoxal-5′-phosphate, 120 μM dithiothreitol, pH 7.4) to a final volume of 400 μL.[35] 3-Mercaptopropionic acid was used as a positive control.

Percentage inhibition (expressed as percent of negative control) was determined by calculating enzyme activity in the presence and absence (vehicle only) of fractions. Methanol vehicle presented no statistically significant interference.

Statistics. Comparisons of biological activities were performed using Sigmaplot (V11.0, Systat Software, Chicago, IL, U.S.A.) using t tests, one-way ANOVAs (Tukey's post hoc), and nonparametric tests (Kruskal−Wallis). All correlations are Pearson Correlation Coefficients (r); 0.8−1 is classed as very strong, 0.6−0.8 strong, and <0.6 weak.

RESULTS

GC Indices and Extractives. Indices for each fraction from final effluents and wood feedstocks are presented in Figure 1. For mills from Brazil, New Zealand (NZ), and Canada Mill D, total GCIs ranged from 20 to 40. For Canada Mills A−C, a number of features standout. First, Canada Mills A and B have total GCIs lower than all other mills (~11), whereas Canada Mill C exhibited the highest total GCI value (57) of the mills surveyed. Apart from Canada Mill C, the highest GCIs for all fractions are associated with SPE-DCM, with SPE-MeOH

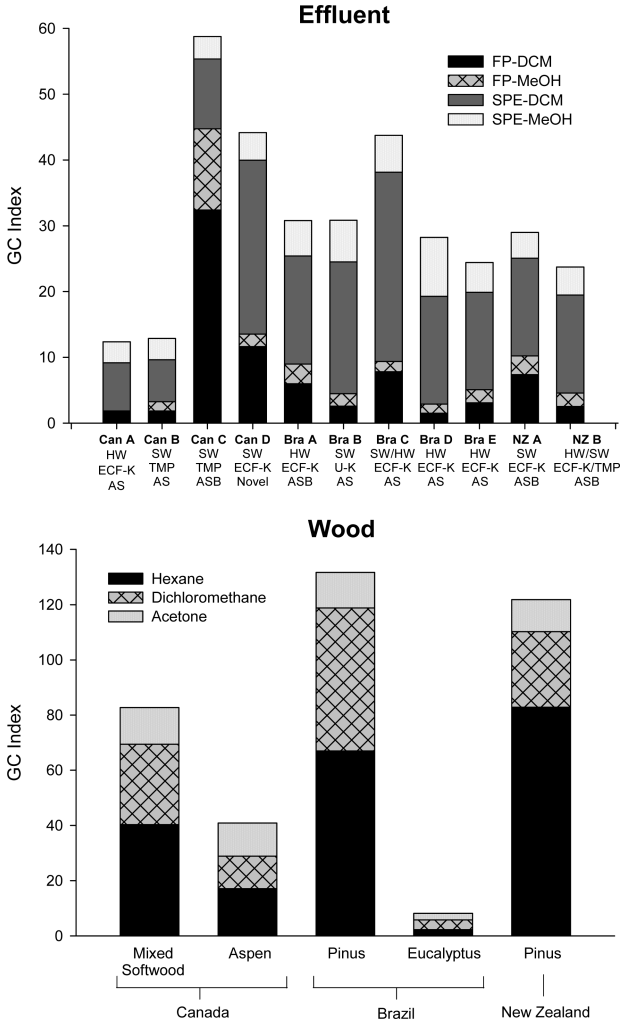


Figure 1. GC indices of extracted final effluents and extracted wood feedstocks from mills in Canada, Brazil, and New Zealand. HW = hardwood, SW = softwood, ECF-K = elemental chlorine free (ClO_2) bleached kraft pulp, TMP = thermo-mechanical pulping, U-K = unbleached kraft, AS = activated sludge secondary treatment system, ASB = aerated stabilization basin treatment system.

typically the next highest. For Canada Mill C, the highest GCI (31) was found with the FP-DCM fraction. For effluents, no obvious relationship between GCIs, wood type, production type, or treatment type was observed. However, GCI was correlated to BOD for kraft mills (0.834, p = 0.001, data not shown).

Fractions from softwood feedstocks produced the highest total GCIs (69−110), followed by the hardwood Canadian Aspen (36), while the lowest total GCI values were associated with Brazilian *Eucalyptus* (8; Figure 1). Within the softwoods examined, GCIs were consistently highest in hexane fractions, with corresponding decreases occurring with increasing polarity of the extraction solvent (dichloromethane followed by acetone).

Total resin acids for combined effluent fractions were <0.06 mg/L with the exception of Canada Mill C (0.36 mg/L), where the bulk (0.28 mg/L) were in the FP-DCM fraction. Total fatty acids for combined effluent fractions were <0.16 mg/L, except for Canada Mill C and NZ Mill A (0.58 and 0.23 mg/L respectively). Like resin acids, the majority of fatty acids were found in FP-DCM fractions (Figure S2, Supporting Information). Softwood chip extracts contained higher resin acids (955−3220 mg/L), while those from hardwoods were much lower (2.1−16.1 mg/L). In all effluents and feedstocks, when resin acids were detected, dehydroabietic acid predominated (effluents <0.08 mg/L except Canada Mill C 0.23 mg/L; softwood chips 575−1375 μg/g; hardwood chips 1.8−8.5 μg/g). The largest proportion of resin acids was present in the most nonpolar extraction solvent (DCM or hexane) for effluents and feedstocks (Figure S2, Supporting Information).

Concentrations of progesterone, AD and ADD, compounds with putative androgenic activity, as well as phenolics and sterols, were below detection limits for all samples. Manool was detected in effluents from Canada Mills C (total manool 10.0 μg/L) and D (29.2 μg/L) and not detected in wood. On the basis of the affinity of manool to the goldfish testis AR,[30] it accounted for <0.05% of the androgenic activities measured for those mills.

Androgenicity and Estrogenicity. All fractions from effluents and wood chips were evaluated for their contents of ligands for androgen (Figure 2) and estrogen (Figure S3, Supporting Information) receptors. All effluents contained some level of androgenicity, as measured by binding to goldfish

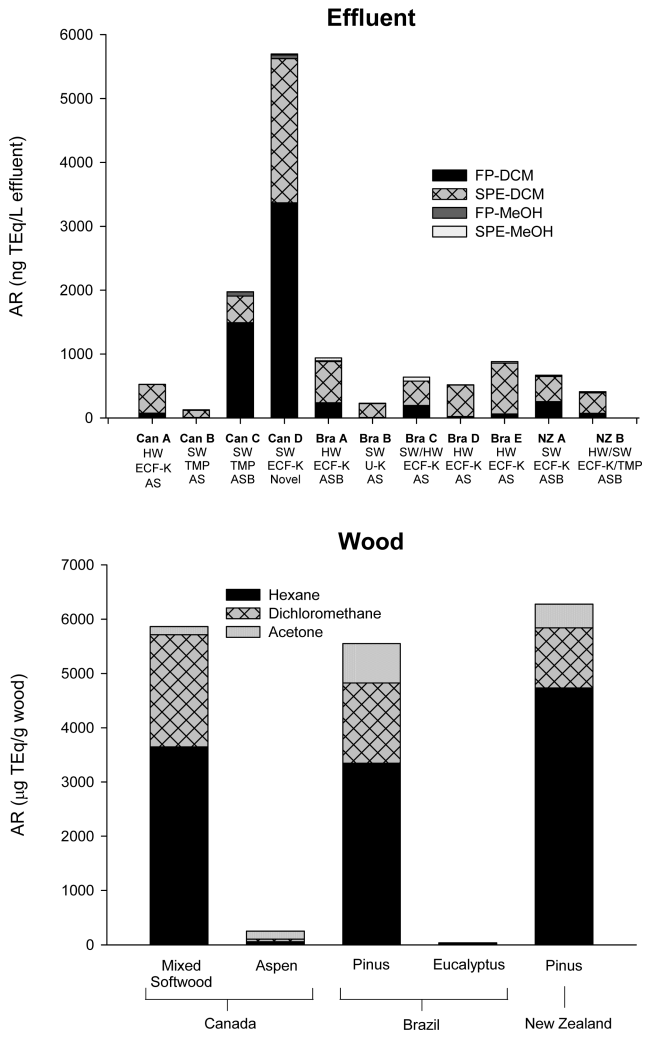


Figure 2. Testosterone equivalents obtained after incubations of final effluent and wood feedstock extracts with goldfish testis androgen receptors (AR). HW = hardwood, SW = softwood, ECF-K = elemental chlorine free (ClO_2) bleached kraft pulp, TMP = thermo-mechanical pulping, U-K = unbleached kraft, AS = activated sludge secondary treatment system, ASB = aerated stabilization basin treatment system.

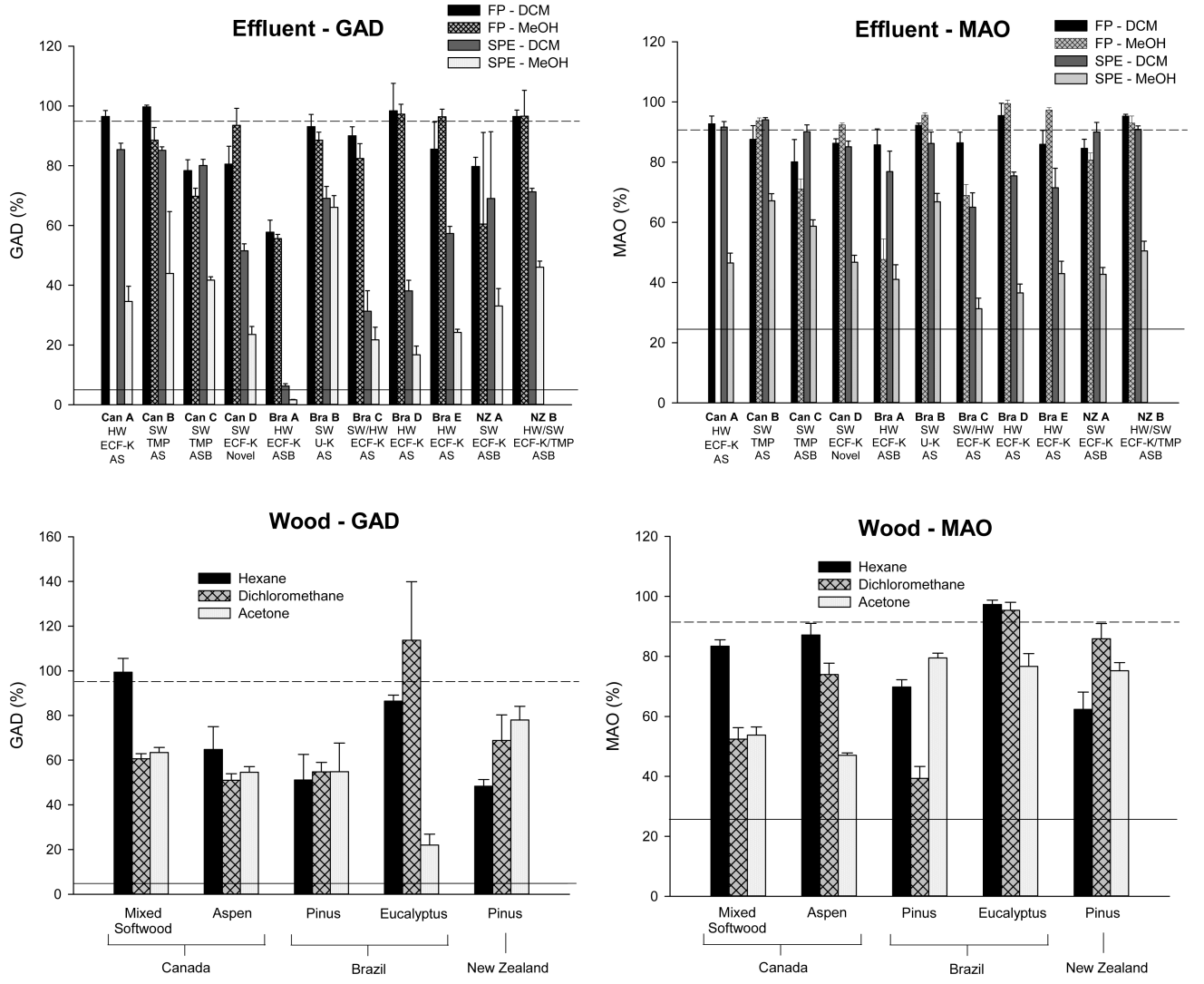


Figure 3. Glutamic acid decarboxylase (GAD) and monoamine oxidase (MAO) neurotransmitter inhibition expressed as a percentage of negative control following incubations with effluent and wood extracts. Dotted lines indicate average activity of method blanks, solid lines indicate average activity of positive control (GAD = 3-mercaptopropionic acid, MAO = clorgyline). All bars are mean ± standard deviation. HW = hardwood, SW = softwood, ECF-K = elemental chlorine free (ClO_2) bleached kraft pulp, TMP = thermo-mechanical pulping, U-K = unbleached kraft, AS = activated sludge secondary treatment system, ASB = aerated stabilization basin treatment system.

testis AR. Canada Mill D, which utilizes a novel treatment system, contained the greatest total AR activity (5697 ng TEq/L, $p < 0.001$), while Canada Mill C (1976 ng TEq/L, $p < 0.001$) had the next highest (Figure 2). For the remaining mills, totals ranged from 129 to 940 ng TEq/L. In all but two cases (Canada Mills C and D), the SPE-DCM fraction contained the highest level of androgenic activity (113−796 ng TEq/L, $p < 0.001$), while the FP-DCM fraction typically contained the majority of any remaining activity (0−255 ng TEq/L; Figure 2). Canada Mills C and D contained the largest activity in the FP-DCM fraction (1491 and 3366 ng TEq/L, respectively, $p < 0.001$), while the SPE-DCM contained lesser levels (417 and 2259 ng TEq/L, respectively, $p < 0.001$). No further relationship between wood, production or treatment type was found.

When comparing the androgenicity of wood fractions, fractions from the Brazilian hardwood *Eucalyptus* (37 μg TEq/g) and Canadian Aspen (252 μg TEq/g) contained much lower AR activity when compared to softwoods from all three countries (5551−6278 μg TEq/g; Figure 2). Androgenic activities were generally greatest in the most nonpolar feedstock fraction (Hexane), with decreased activity in more polar extraction solvents.

Very little estrogenic (ER) activity was evident using the YES assay, with nearly all results below detection limits (10.4 ng/L effluent; 4.1 ng/g wood; Figure S3, Supporting Information). Only three fractions showed any ER activity, and all were from Brazilian mills. Two were SPE-DCM fractions (Brazil Mills B and D), and one was a FP-MeOH fraction (Brazil Mill E). No ER activity was observed in any wood fractions.

Neurotransmitter Enzymes. All effluents and wood feedstocks were evaluated for their ability to inhibit two neurotransmitter enzymes, GAD and MAO, with every effluent and wood sample containing inhibitors of both enzymes (Figure 3). For all effluents, SPE-MeOH fractions caused the greatest inhibition for MAO, and in eight of the eleven mills for GAD ($p < 0.001$). For the remaining three (Brazil Mills A, B, and C), the highest GAD inhibition was contained in both SPE fractions ($p < 0.001$). Generally, FP fractions for all mills did not inhibit GAD or MAO. Brazil Mill A stands out by inhibiting GAD the greatest, where all fractions significantly interfered with the

enzyme, although this was not reflected with MAO. For both GAD and MAO, independent of country, wood feedstock, pulping process, and treatment type, the seven kraft ECF mills caused the greatest inhibitions ($p < 0.002$; Figure 4). In the case

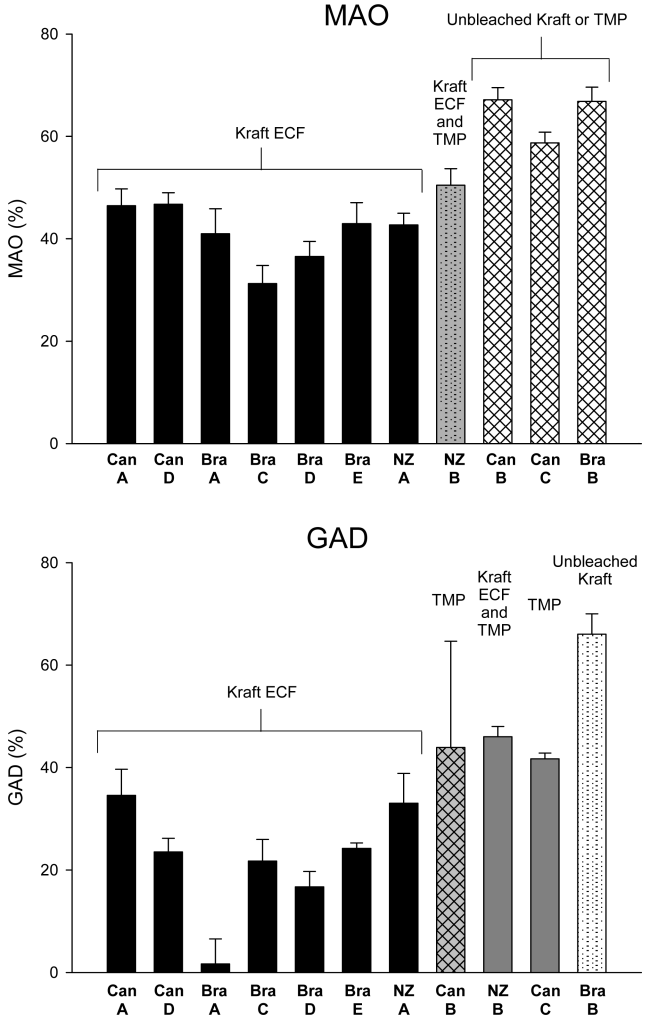


Figure 4. Monoamine oxidase (MAO) and glutamic acid decarboxylase (GAD) neurotransmitter inhibition expressed as a percentage of negative control following incubations with effluent SPE-MeOH fractions. All bars are mean ± standard deviation. Groupings are based on statistically significant differences (ANOVA, $p < 0.002$), except for GAD Can B vs kraft ECF ($p = 0.648$), NZ B and Can C ($p = 0.979$), Bra B ($p > 0.798$) because of an assay problem with Can C replicates. Can = Canada, Bra = Brazil, NZ = New Zealand.

of MAO, New Zealand Mill B, which produces ECF kraft and thermomechanical (TMP) pulp, showed an intermediate potency ($p < 0.001$), while the least potent mills of this study were the unbleached kraft and two TMP mills ($p < 0.002$). For GAD, New Zealand Mill B and Canada Mill C (TMP only) were of intermediate potency ($p < 0.001$), while the unbleached kraft mill, Brazil B, was the least potent ($p < 0.001$).

While neurotransmitter assays showed that all wood feedstocks contain GAD and MAO inhibitors (Figure 3), no trends between species, countries, and fractions are evident.

Parameters Related to In Vitro Activities. Relationships between bioactivities, effluent and wood chemistry, and production metrics were assessed using Pearson correlations (Tables S2 and S3, Supporting Information), with highlights detailed below.

Relationships with AR Activity. With effluents, the FP-DCM fractions produced a very strong correlation of 0.995 ($p < 0.001$), when the mill utilizing novel effluent treatment (Canada Mill D) was excluded (Table S2, Supporting Information). When the solvent types were pooled, a strong correlation of 0.784 ($p < 0.001$) was noted for DCM (Figure S5, Supporting Information), again when Canada Mill D was excluded. The MeOH extractions showed a weak significant correlation of 0.447 ($p = 0.042$).Since all effluent fractions, regardless of production, wood type and treatment (activated sludge or aerated lagoons), were highly correlated, the novel treatment system used by Canada Mill D is justified as an outlier. No correlations were observed between effluent total resin or fatty acids and AR binding.

The AR ligand contents of wood were also strongly correlated to wood GCIs (0.9210, $p < 0.001$), which was driven by fractions extracted from softwoods (Figure S5, Supporting Information, 0.890, $p < 0.001$). AR activity was also very strongly correlated to total resin acids (0.920, $p < 0.001$) and strongly to total fatty acids (0.792, $p < 0.001$; Table S3, Supporting Information).

Relationships with GAD and MAO Inhibition. Effluent MAO and GAD inhibition were strongly correlated with GCI when extractions were pooled by solvent type. Pooling all MeOH fractions (Table S2, Figure S6, Supporting Information), strongly significant negative correlations are observed between GCI and MAO (−0.741, $p < 0.001$), as well as between GCI and GAD (−0.753, $p < 0.001$), when one mill (Canada Mill C) is excluded. These correlations can be justified by the high effluent TSS of this mill (Supporting Information Table S1). With pooled DCM extractions (Table S2, Figure S6, Supporting Information), a strongly significant negative correlation between GCI and MAO inhibition (−0.657, $p < 0.001$), and GCI and GAD inhibition (−0.629) was noted. No relationship with MAO or GAD and effluent total resin or total fatty acids was observed. Additionally, no correlations were found with MAO and GAD inhibition and GCI, FA, or RA for wood feedstocks.

DISCUSSION

In this study, we sought to benchmark the current global potential of mill effluents and wood feedstocks to affect fish reproduction by comparing samples from three major pulp-producing nations, where each facility was in regulatory compliance. Mills included those that pulped hardwoods and softwoods, used TMP, ECF-, and unbleached kraft pulping, and employed aerated lagoons, activated sludge and one novel treatment system. We showed that treated effluents from all mills contained ligands for the goldfish testis AR and inhibitors of two neurotransmitter enzymes, however, little estrogenicity was detected. Factors found to influence in vitro activities included a novel effluent treatment system, high TSS, and pulping and bleaching processes. Extracts of wood feedstocks showed similar contents of biologically active substances, however, some large distinctions between hardwoods and softwoods were noted. A GCI was shown to predict AR ligand content, MAO inhibition, and to a lesser extent GAD inhibition, in effluents, and the AR ligand contents of softwoods.

The GCI used was modified from other studies[8,18] by distinguishing particulate bound from dissolved compounds, a range of polarities, and for the first time to profile wood extracts. Building on its ability to predict effects on fathead minnow egg production,[8] we sought to evaluate if the GCI

could also predict the in vitro activities of mill effluents. For kraft mill effluents, total GCI has been strongly correlated to BOD,[8] which was also the case when all kraft mills in this study were considered. Because of the complexity of effluent and wood extracts, a GCI further offers the ability to compare extracts from multiple sources without determining the identities of the actual constituents. In general, effluent SPE-DCM fractions had the highest GCIs, except Canada Mill C (FP-DCM). Canada Mill C also had the highest TSS (>12 fold, Supporting Information Table S1), which may have contributed to it having the highest GCI total of all mills (Figure 1). Overall, little distinction in GCI can be observed between wood type, pulping or bleaching types, or biological treatment type in effluent extracts.

Since very polar or very large compounds are not included in the GCI, the development of an index using liquid chromatography may help in predicting activity associated with these compounds. Such compounds would be found in methanol effluent extracts, which were darkly colored, yet exhibited generally low GCIs. It is worth noting, however, that GCIs were still predictive of MAO inhibition in these same extracts.

Because of their degradability, effluent resin and fatty acids were low, with the exception of Canada Mill C, which had the highest TSS, and would contain particulate-bound acids. Softwood chip extracts contained significantly higher resin and fatty acids and GCI values than for hardwoods, as expected.[36] It is noteworthy that these wood-derived differences are not reflected in effluents from hardwood versus softwood mills. It is likely that a large percentage of the detectable material by GC is significantly metabolized during biological treatment.[37]

Ligands for the AR were consistently detected in all final effluents and predominantly in softwood chip extracts (Figure 2). In contrast, very low YES activity was detected in only three Brazilian mills, and not wood chip extracts. In one of these mills, Brazil Mill A, sanitary waste from the site is also treated in the treatment system, which may account for some of the YES activity. The overall lack of estrogenic activity is surprising given the evidence for Canadian[38] and South American[13,15] mill effluents and experiments with stilbenes, plant sterols, and resin acids.[39−41] It is however, consistent with studies at both New Zealand mills[12] included in this study. That estrogenic activity was detected in three Brazilian mills (Supporting Information Figure S3) coincides with the strength of the in vitro activity noted recently in Chilean mill effluent extracts from both *Eucalyptus* and *Pinus*.[14,26] The lack of estrogenic response in this study may be related to the in vivo aromatization of AR ligands, which would not be detectable using the YES assay.[42] The estrogenic activity of selected fractions is currently being further examined using rainbow trout.

Excluding Canada Mills C and D, little overall difference in AR activity is exhibited between pulping and bleaching types, wood types, and biological treatment systems. Canada Mill D, which utilizes a novel treatment system, contained >8-fold the total average androgenicity of the other mills. Canada Mill C, which had the highest TSS levels, was the mill that contained the highest AR levels in the FP-DCM fraction (Figure 2). This is consistent with previous studies from New Zealand that showed filtering the solids removed androgenic activity.[5] These two mills illustrate the role that conventional biotreatment and subsequent solids removal has on reducing the overall androgenicity of final effluents.

We found very strong positive correlations of GCI to AR binding activity for both wood and effluent extracts. In all but two cases, effluent androgenicity was predominantly associated with the SPE-DCM fraction, with the majority of the remainder of activity in the FP-DCM fraction. This indicates AR ligands are intermediate to nonpolar, consistent with effects-directed studies of androgens in Canadian kraft chemical recovery condensates,[30] and bioaccumulation studies at Canadian mills.[43,44]

Similar to effluents, most of the androgenic activity for wood was in the nonpolar fractions. Unlike effluent fractions, wood vextracts showed a large difference between hardwoods and softwoods with hardwoods having negligible AR activity (Figure 2). This discrepancy was also reflected in the GCI (Figure 1) and total resin acid (Figure S2, Supporting Information) values. The lack of a difference in effluents between mills pulping softwoods or hardwoods suggests that (a) hardwood mills produce androgenic compounds at some point during pulping, bleaching, or subsequent biotreatment; (b) the androgenic compounds are significantly degraded in softwood mills; or (c) some androgenic compounds found in both types of wood are of similar nature and nondegraded.

Because of the complexity of final effluents, the actual causative androgens still remain elusive, despite many studies.[45] Androgens associated with wood and effluents, ADD, AD, and progesterone[27−29] were not detected in this study, and thus are not involved in the androgenic activities we observed. Recently, the diterpene manool was discovered as a ligand for the goldfish testis AR and a major contributor to the androgenicity of Canadian kraft chemical recovery condensates.[30] In this study, manool was only detected in Canada Mills C and D effluents, accounting for <0.05% of those mills' AR activities. Since the structure of manool is similar to resin acids, which are readily biodegradable,[37] it can be implied that effluent biotreatment is effective in the removal of manool and other structurally analogous AR ligands. Collectively, based on the results of this study, it appears that effluent biotreatment has beneficial effects toward reducing androgen discharges, especially for softwood mills.

Neuroendocrine activity, measured as MAO and GAD inhibition, was observed in all effluents and wood chips from the three countries studied. Effluents from the seven ECF kraft mills produced the greatest MAO and GAD inhibition relative to the one unbleached kraft and two TMP mills. While this suggests that kraft ECF bleaching generates more compounds affecting the neuroendocrine system, implications for the pulp and paper sector necessitate further examination. Contrary to AR activity, neuroendocrine activity was greatest in the most polar effluent extracts. Indeed, there was no correlation between AR and either MAO or GAD ($p > 0.05$, data not shown), confirming different classes of compounds are involved. This is not surprising, given the structural conformations required between these three end points, and that each represents a different mode of action affecting fish reproduction. Given that the most polar fractions for wood did not inhibit GAD or MAO differently from nonpolar extracts, it would appear that pulping, bleaching and effluent treatment play a role in the generation of neurotransmitter inhibitors, as evidenced above for ECF kraft.

Previous work[20] showed that for final treated effluent at a Canadian TMP mill, MAO inhibition (65%) remained associated with the water fraction following extraction using polyvinylpolypyrrolidone powder. In contrast, we show that it is possible to remove neuroactive compounds from effluents using SPE. Both TMP mills of this study showed similar MAO inhibition of

60−65% in the most polar fraction (SPE-MeOH). Interestingly, while no GAD inhibition was observed by Basu et al.,[20] we found it not only at both Canadian TMP mills, but in all mill effluents from Canada, Brazil, and New Zealand. Since we observed the most potent GAD inhibitors in SPE fractions, the polar compounds recovered by SPE were possibly retained on the polyvinylpolypyrrolidone powder used for effluent extractions.[20] These results all indicate that GAD and MAO inhibitors are very polar, water-soluble, and therefore, readily bioavailable. This supports the hypothesis of neurotransmitter involvement in the rapid onset and subsequent recovery of some reproductive end points, such as egg production[8] and sex steroid levels.[10]

In conclusion, this study determined the prevalence of androgenic and neuroactive compounds in all effluents and wood feedstocks sampled in Brazil, Canada, and New Zealand. No differences in bioactivities could be discerned on the basis of effluent or wood country of origin. Differences in bioactivities between hardwood and softwood chip extracts were not reflected in effluents generated from these feedstocks, implying that pulping, bleaching, and treatment mitigate bioactive wood components irrespective of locale. This highlights the difficulty in identifying causative agents, and does not discount the possibility that other process chemicals are important for effluent related effects. The difference in bioactivity between wood and effluents highlights the impact that mill operating conditions can have in final effluent biological activities. Effluent treatment type (novel vs ASB/AS) and effluent solids management were important in controlling androgenic compound releases, while ECF kraft was associated with increased inhibition of GAD and MAO. The GCI, incorporating solids extraction and differentiation of compound polarity, was predictive of the majority of in vitro activity. On the basis of this study, we would predict effluents where no monitoring is conducted, to also affect fish reproduction. It is therefore recommended that monitoring programs, analogous to the Canadian EEM program, be implemented in these jurisdictions.

■ ASSOCIATED CONTENT

Supporting Information

Summary of effluent sample characteristics, recovery of effluent extractives, correlations for effluent fractions, correlations for wood extracts, total resin and fatty acid concentrations, estrogen receptor binding assay, gas chromatographic index, relationship between GCI and goldfish testis androgen receptor binding, and relationship between GCI and MAO. This information is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Address: National Water Research Institute, Environment Canada 867 Lakeshore Rd, Burlington, ON, Canada. E-mail: craig.milestone@ec.gc.ca.

■ ACKNOWLEDGMENTS

The authors gratefully acknowledge the participating mills for providing samples and mill information, the staff in Aplysia (Brazil), Scion (New Zealand), FPInnovations (Canada), and Environment Canada. Jim Bennett is also acknowledged for his photo used in the Table of Contents and Abstract.

■ REFERENCES

(1) Sodergren, A.; Bengtsson, B. E.; Jonsson, P.; Lagergren, S.; Larson, A.; Olsson, M.; Renberg, L. Summary of results from the Swedish Project Environment Cellulose. *Water Sci. Technol.* **1988**, *20* (1), 49−60.

(2) Hewitt, L. M.; Kovacs, T. G.; Dubé, M. G.; MacLatchy, D. L.; Martel, P. H.; McMaster, M. E.; Paice, M. G.; Parrott, J. L.; van den Heuvel, M. R.; Van Der Kraak, G. J. Altered reproduction in fish exposed to pulp and paper mill effluents: Roles of individual compounds and mill operating conditions. *Environ. Toxicol. Chem.* **2008**, *27* (3), 682−697.

(3) van den Heuvel, M. R.; Ellis, R. J.; Smith, M. A.; Finley, M.; Stuthridge, T. Review of reproductive−endocrine effects of a New Zealand pulp and paper mill effluent. In *Pulp and Paper Mill Effluent Environmental Fate and Effects*; Borton, D. L., Hall, T. J., Fisher, R. P., Thomas, J. F., Eds.; Destech Publications: Lancaster, PA, U.S.A., 2004; pp 55−66.

(4) McMaster, M. E.; Hewitt, L. M.; Parrott, J. L. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: Field studies and mechanistic research. *J. Toxicol. Environ. Health, Part B.* **2006**, *9* (4), 319−339.

(5) Ellis, R. J.; van den Heuvel, M. R.; Smith, M. A.; McCarthy, L. H.; Stuthridge, T. R.; Dietrich, D. R. In vivo and in vitro assessment of the androgenic potential of a pulp and paper mill effluent. *Environ. Toxicol. Chem.* **2003**, *22* (7), 1448−1456.

(6) Parrott, J. L.; McMaster, M. E.; Hewitt, L. M. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: Development and application of fish bioassays. *J. Toxicol. Environ. Health, Part B.* **2006**, *9* (4), 297−317.

(7) Martel, P.; Kovacs, T.; Voss, R. Survey of pulp and paper mill effluents for their potential to affect fish reproduction. In *Pulp and Paper Mill Effluent Environmental Fate and Effects*; Borton, D. L., Hall, T. J., Fisher, R. P., Thomas, J. F., Eds.; Destech: Lancaster, PA, U.S.A., 2004; pp 78−91.

(8) Kovacs, T. G.; Martel, P. H.; O'Connor, B. I.; Parrott, J. L.; McMaster, M. E.; Van Der Kraak, G. J.; MacLatchy, D. L.; van den Heuvel, M. R.; Hewitt, L. M. Kraft mill effluent survey: Progress toward best management practices for reducing effects on fish reproduction. *Environ. Toxicol. Chem.* **2011**, *30* (6), 1421−1429.

(9) Larsson, D. G. J.; Förlin, L. Male-biased sex ratios of fish embryos near a pulp mill: Temporary recovery after a short-term shutdown. *Environ. Health Perspect.* **2002**, *110* (8), 739−742.

(10) Munkittrick, K. R.; Van Der Kraak, G. J.; McMaster, M. E.; Portt, C. B. Response of hepatic MFO activity and plasma sex steroids to secondary-treatment of bleached kraft pulp-mill effluent and mill shutdown. *Environ. Toxicol. Chem.* **1992**, *11* (10), 1427−1439.

(11) Lowell, R. B.; Ring, B.; Patershank, G.; Walker, S. L.; Trudel, L.; Hedley, K. *National Assessment of the Pulp and Paper Environmental Effects Monitoring Data: Findings from Cycles 1 through 3*, NWRI Scientific Assessment Report Series 5; National Water Research Institute: Burlington, ON, Canada, 2005.

(12) van den Heuvel, M. R.; Slade, A. H.; Landman, M. J. Summary of a decade of research on the effects of a New Zealand pulp and paper millon reproduction in fishes. *Water Qual. Res. J. Can.* **2010**, *45* (2), 123−135.

(13) Orrego, R.; Moraga-Cid, G.; Gonzalez, M.; Barra, R.; Valenzuela, A.; Burgos, A.; Gavilan, J. E. Reproductive, physiological, and biochemical responses in juvenile female rainbow trout (*Oncorhynchus mykiss*) exposed to sediment from pulp and paper mill industrial discharge areas. *Environ. Toxicol. Chem.* **2005**, *24* (8), 1935−1943.

(14) Chamorro, S.; Hernádez, V.; Monsalvez, E.; Becerra, J.; Mondaca, M. A.; Piña, B.; Vidal, G. Detection of estrogenic activity from kraft mill effluents by the yeast estrogen screen. *Bull. Environ. Contam. Toxicol.* **2010**, *84* (2), 165−169.

(15) Orrego, R.; Burgos, A.; Moraga-Cid, G.; Inzunza, B.; Gonzalez, M.; Valenzuela, A.; Barra, R.; Gavilan, J. E. Effects of pulp and paper mill discharges on caged rainbow trout (*Oncorhynchus mykiss*): Biomarker responses along a pollution gradient in the Biobio River, Chile. *Environ. Toxicol. Chem.* **2006**, *25* (9), 2280−2287.

(16) Chiang, G.; McMaster, M. E.; Urrutia, R.; Saavedra, M. F.; Gavilan, J. F.; Tucca, F.; Barra, R.; Munkittrick, K. R. Health status of native fish (*Percilia gillissi* and *Trichomycterus areolatus*) downstram of the discharge of effluent from a tertiary treated elemental chlorine free pulp mill in Chile. *Environ. Toxicol. Chem.* **2011**, *30* (8), 1793−1809.
(17) Munkittrick, K. R.; McMaster, M. E.; McCarthy, L. H.; Servos, M. R.; Van Der Kraak, G. J. An overview of recent studies on the potential of pulp-mill effluents to alter reproductive parameters in fish. *J. Toxicol. Environ. Health, Part B.* **1998**, *1* (4), 347−371.
(18) Martel, P. H.; Kovacs, T. G.; O'Connor, B. I.; Semeniuk, S.; Hewitt, L. M.; MacLatchy, D. L.; McMaster, M. E.; Parrott, J. L.; van den Heuvel, M. R.; Van Der Kraak, G. J. Effluent monitoring at a bleached kraft mill: Directions for best management practices for eliminating effects on fish reproduction. *J. Environ. Sci. Health, Part A* **2011**, *46*, 833−843.
(19) van den Heuvel, M. R.; Martel, P. H.; Kovacs, T. G.; MacLatchy, D. L.; Van Der Kraak, G. J.; Parrott, J. L.; McMaster, M. E.; O'Connor, B. I.; Melvin, S. D.; Hewitt, L. M. Evaluation of short-term fish reproductive bioassays for predicting effects of a Canadian bleached kraft mill effluent. *Water Qual. Res. J. Can.* **2010**, *45* (2), 175−186.
(20) Basu, N.; Ta, C. A.; Waye, A.; Mao, J. Q.; Hewitt, M.; Arnason, J. T.; Trudeau, V. L. Pulp and paper mill effluents contain neuroactive substances that potentially disrupt neuroendocrine control of fish reproduction. *Environ. Sci. Technol.* **2009**, *43* (5), 1635−1641.
(21) Waye, A.; Trudeau, V. L. Neuroendorcrine disruption: More than hormones are upset. *J. Toxicol. Environ. Health, Part B* **2011**, *14*, 270−291.
(22) Popesku, J. T.; Tan, E. Y. Z.; Martel, P. H.; Kovacs, T. G.; Rowan-Carroll, A.; Williams, A.; Yauk, C.; Trudeau, V. L. Gene expression profiling of the fathead minnow (Pimephales promelas) neuroendocrine brain in response to pulp and paper mill effluents. *Aquat. Toxicol.* **2010**, *99* (3), 379−388.
(23) Dufour, S.; Weltzien, F. A.; Sebert, M. E.; Le Belle, N.; Vidal, B.; Vernier, P.; Pasqualini, C. Dopaminergic inhibition of reproduction in teleost fishes: Ecophysiological and evolutionary implications. *Ann. N.Y. Acad. Sci.* **2005**, *1040*, 9−21.
(24) Trudeau, V. L. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Camssius auratus*. *Rev. Reprod.* **1997**, *2* (1), 55−68.
(25) Popesku, J. T.; Martyniuk, C. J.; Mennigen, J.; Xiong, H.; Zhang, D.; Xia, X.; Cossins, A. R.; Trudeau, V. L. The goldfish (*Carassius auratus*) as a model for neuroendocrine signaling. *Mol. Cell. Endocrinol.* **2008**, *293* (1−2), 43−56.
(26) Orrego, R.; Guchardi, J.; Hernandez, V.; Krause, R.; Roti, L.; Armour, J.; Ganeshakumar, M.; Holdway, D. Pulp and paper mill effluent treatments have differential endocrine-disrupting effects on rainbow trout. *Environ. Toxicol. Chem.* **2009**, *28* (1), 181−188.
(27) Parks, L. G.; Lambright, C. S.; Orlando, E. F.; Guillette, L. J.; Ankley, G. T.; Gray, L. E. Masculinization of female mosquitofish in kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicol. Sci.* **2001**, *62* (2), 257−267.
(28) Carson, J. D.; Jenkins, R. L.; Wilson, E. M.; Howell, W. M.; Moore, R. Naturally occurring progesterone in loblolly pine (*Pinus taeda* L.): A major steroid precursor of environmental androgens. *Environ. Toxicol. Chem.* **2008**, *27* (6), 1273−1278.
(29) Jenkins, R.; Angus, R. A.; McNatt, H.; Howell, W. M.; Kemppainen, J. A.; Kirk, M.; Wilson, E. M. Identification of androstenedione in a river containing paper mill effluent. *Environ. Toxicol. Chem.* **2001**, *20* (6), 1325−1331.
(30) Scott, P. D.; Milestone, C. B.; Smith, S.; MacLatchy, D. L.; Hewitt, L. M. Isolation and identification of ligands for the goldfish testis androgen receptor in chemical recovery condensates from a Canadian bleached kraft pulp and paper mill. *Environ. Sci. Technol.* **2011**, *45* (23), 10226−10234.
(31) Voss, R. H.; Rapsomatiotis, A. An improved solvent-extraction based procedure for the gas chromatographic analysis of resin and fatty acids in pulp mill effluents. *J. Chromatogr. A* **1985**, *346* (C), 205−214.
(32) Wells, K.; Van Der Kraak, G. J. Differential binding of endogenous steroids and chemicals to androgen receptors in rainbow trout and goldfish. *Environ. Toxicol. Chem.* **2000**, *19* (8), 2059−2065.
(33) Gaido, K. W.; Leonard, L. S.; Lovell, S.; Gould, J. C.; Babaï, C. J.; Portier, C. J.; Donnel, D. P. M. Evaluation of chemicals with endocrine modulating activity in yeast based steroid hormone receptor gene transcription assay. *Toxicol. Appl. Pharmacol.* **1997**, *143*, 205−212.
(34) Basu, N.; Scheuhammer, A. M.; Evans, R. D.; O'Brien, M.; Chan, L. H. M. Cholinesterase and monoamine oxidase activity in relation to mercury levels in the cerebral cortex of wild river otters. *Hum. Exp. Toxicol.* **2007**, *26* (3), 213−220.
(35) Awad, R.; Levac, D.; Cybulska, P.; Merali, Z.; Trudeau, V. L.; Arnason, J. T. Effects of traditionally used anxiolytic botanicals on enzymes of the γ-aminobutyric acid (GABA) system. *Can. J. Physiol. Pharmacol.* **2007**, *85* (9), 933−942.
(36) Sjöström, E. *Wood Chemistry: Fundamentals and Applications*, 2 ed.; Academic Press: London, 1993.
(37) Liss, S. N.; Bicho, P. A.; Saddler, J. N. Microbiology and biodegradation of resin acids in pulp mill effluents: A minireview. *Can. J. Microbiol.* **1997**, *43* (7), 599−611.
(38) Kovacs, T.; Martel, P.; Ricci, M.; Michaud, J.; Voss, R. Further insights into the potential of pulp and paper mill effluents to affect fish reproduction. *J. Toxicol. Environ. Health* **2005**, *68* (19), 1621−1641.
(39) Mellanen, P.; Petanen, T.; Lehtimaki, J.; Makela, S.; Bylund, G.; Holmbom, B.; Mannila, E.; Oikari, A.; Santti, R. Wood-derived estrogens: Studies in vitro with breast cancer cell lines and in vivo in trout. *Toxicol. Appl. Pharmacol.* **1996**, *136* (2), 381−388.
(40) MacLatchy, D. L.; Van Der Kraak, G. J. The phytoestrogen beta-sitosterol alters the reproductive endocrine status of goldfish. *Toxicol. Appl. Pharmacol.* **1995**, *134* (2), 305−312.
(41) Parrott, J. L.; Kohli, J.; Sherry, J. P.; Hewitt, L. M. In vivo and in vitro mixed-function oxygenase activity and vitellogenin induction in fish and in fish and rat liver cells by stilbenes isolated from scotch pine (*Pinus sylvestris*). *Arch. Environ. Contam. Toxicol.* **2011**, *60* (1), 116−123.
(42) Raghuveer, K.; Senthilkumaran, B. Identification of multiple dmrt1s in catfish: localization, dimorphic expression pattern, changes during testicular cycle and after methyltestosterone treatment. *J. Mol. Endocrinol.* **2009**, *42* (5−6), 437−448.
(43) Hewitt, L. M.; Pryce, A. C.; Parrott, J. L.; Marlatt, V.; Wood, C.; Oakes, K.; Van Der Kraak, G. J. Accumulation of ligands for aryl hydrocarbon and sex steroid receptors in fish exposed to treated effluent from a bleached sulfite/groundwood pulp and paper mill. *Environ. Toxicol. Chem.* **2003**, *22* (12), 2890−2897.
(44) Hewitt, L. M.; Parrott, J. L.; Wells, K. L.; Calp, M. K.; Biddiscombe, S.; McMaster, M. E.; Munkittrick, K. R.; Van Der Kraak, G. J. Characteristics of ligands for the Ah receptor and sex steroid receptors in hepatic tissues of fish exposed to bleached kraft mill effluent. *Environ. Sci. Technol.* **2000**, *34* (20), 4327−4334.
(45) Hewitt, L. M. Effects-directed studies of pulp and paper mill effluents. In *Effect-Directed Analysis of Complex Environmental Contamination*; Brack, W., Ed.; Springer-Verlag: Berlin, 2011; Vol. *15*, pp 267−283.