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A Decade of Research on the Environmental Impacts of Pulp and Paper Mill Effluents in Canada (1992 – 2002)

M.E. McMaster, J.L. Parrott and L.M. Hewitt
National Water Research Institute
Environment Canada

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1. Executive Summary

In the late 1980's, pulp and paper production worldwide became an area of increased environmental scrutiny by the public as dioxins and furans in effluents and paper products were found. At the same time, results from Swedish studies in the early 1980s provided some of the first evidence that effluents from some pulp mills were capable of inducing toxic responses in fish at very low concentrations in the receiving environment. In response to these studies, a number of Canadian studies were initiated to determine whether similar responses were seen in fish downstream of pulp mills in Canada. Reproductive alterations were identified at a number of locations, however not all studies demonstrated similar reproductive effects in the receiving environment. A new regulatory package for the pulp and paper industry was then introduced which set new levels for BOD, TSS and acute lethality as well as limits for dioxins and furans. In order to meet the new regulatory limits, a number of process and treatment changes were required by the industry however it was not known whether these upgrades would also improve the reproductive responses demonstrated in fish at a number of locations.

Subsequent to the regulatory package, the Minister of the Environment announced a large collaborative research program involving government, industry and academia. The original objectives of the research program were to 1) identify the causative organochlorine compounds, 2) determine how to eliminate them from the process, and 3) determine the short-term and long-term environmental effects of these compounds. Results from this research program along with feedback from the Canadian Environmental Effects Monitoring program (within the new regulations) would then be used to define what additional control actions may be necessary. The following is a review of the research conducted over the last 10 years following the implementation of the 1992 regulatory package. It is separated into three specific sections of research, 1) Field Studies and Mechanistic Research, 2) Development and Application of Bioassays, and 3) Characterization of Bioactive Chemicals.

Field Studies and Mechanistic Research

Field studies first confirmed the Swedish results at a number of primary treated bleached kraft mills in Canada. These studies identified a number of reproductive alterations in fish including reduced gonadal development, increased age to maturation, reduced expression of secondary sex characteristics, reduced fecundity and reductions in circulating levels of the major biologically active reproductive steroid hormones. Continued research studies then determined that reproductive impacts in fish were not limited to mills that used chlorine in the bleaching process and that these effects were still present at some mills that employed secondary effluent treatment. These findings were important as it suggested that other mills in Canada had the potential of causing these types of responses in fish however other studies failed to identify responses in fish downstream of some of the more modern facilities. Detailed mechanistic studies at one site identified reproductive impacts at a number of locations within the hypothalamic-pituitary-gonadal axis controlling reproductive function. These included impacts on pituitary function, ovarian steroid biosynthetic capacity, and peripheral steroid metabolism. Further study identified alterations in the gonadal steroid biosynthetic capacity as the major site of reproductive action. Alterations in a number of steroidogenic enzymes within the biosynthetic pathway were found, although the major impact appeared to be on the availability of cholesterol substrate for steroid production. The alterations in steroid productive capacity led to reductions in circulating levels of reproductive steroids and the cascade of other reproductive changes.

Studies continued at a number of locations and some signs of recovery were demonstrated following both process and treatment changes in response to the new regulations. This recovery however was not complete as changes were still evident relative to comparable

reference locations. The specific role of the various process and treatment changes in the reproductive recovery was unknown as numerous changes were made over time at the mills. The development of bioassays that could predict these reproductive effects was initiated, and then used to aid in identifying which process streams and treatment changes were responsible with confirmation using field studies. There was also still a great deal of emphasis on the difficulties in interpreting results from these field studies and a number of research projects were developed to address these concerns. Some of the concerns included the influence of natural variability, the selection of reference sites, the mobility of sentinel species and the influence of potential confounding factors.

As part of the new regulatory framework, an Environmental Effects Monitoring program was also initiated, which required mills to conduct receiving water fish and benthic invertebrate studies. Following the second cycle of the program, a national assessment was conducted identifying national response patterns in both benthos and fish endpoints. The finding of an average national response pattern consistent with the early studies at a limited number of pulp and paper mills suggested that there were still concerns regarding pulp and paper mill effects on the reproduction of fish via alterations in levels of sex steroid hormones. At the same time, the measured increases in energy use and storage suggested that there was sufficient food for the fish, consistent with the eutrophication responses evident in the invertebrate studies.

Development and Application of Bioassays

Laboratory tests have been used to answer many of the regulatory and research questions that have arisen related to the effects of pulp and paper mill effluents on aquatic biota. Acute, short-term, laboratory tests have been used to assess final effluents and to track changes in effluent quality over time. Short exposures of fish and invertebrates form part of Federal and Provincial effluent discharge regulations for pulp and paper mills and other industries. Short-term laboratory tests have clearly shown the improvement in final effluent quality through the installation of secondary treatment of effluent following the 1992 regulatory package.

Laboratory assays of pulp and paper mill effluents have been used to assess whether currently released effluent is impacting biota. This is an important function of the laboratory tests, the discrimination of the effects of today's effluent from historically contaminated sediments in receiving environments. This isolation of the effects of current effluent is vital, as most of Canada's pulp and paper mills have been operating and discharging for over 30 years.

In an effort to predict impacts on wild fish, laboratory bioassays have been developed to examine the responses seen in fish captured in pulp and paper mill receiving environments: MFO induction and steroid depression. Exposures of fish in the laboratory have been used to identify pulp mill-related chemicals that induce MFO and suppress steroids. Short-term laboratory exposures of fish have been used to assess responses of fish to pulp and paper mill effluents and to specific components of pulp and paper mill processes (such as plant compounds, and black liquors).

These short-term bioassays have enabled specific responses, such as MFO induction or steroid depression, to be linked to mill process streams or waste products. Assessment of MFO induction and steroid reduction caused by pulp and paper mill effluents has enabled comparison of types of mill processes, specific wood furnish, and effluent treatment. It appears that MFO induction and steroid depression are found in fish exposed to effluent from a variety of mills types, with and without chlorine bleaching, in hardwood and softwood pulping facilities, before and after effluent treatment.

New genomic techniques have contributed to the ability of laboratory bioassays to detect changes associated with exposure to pulp and paper mill effluents. The development of chromosomal markers to determine the genetic sex of some fish species has led to some novel findings: after 1-month exposure to high concentrations of bleached kraft mill effluent, some male fish had ovaries. Advanced genetic fingerprinting techniques have shown that pulp and paper mill effluent decreases sex steroids in exposed fish, but the gene expression pattern is different to estradiol. These genetic technologies may lead to the development of shorter bioassays that are linked to reproductive effects.

Longer-term, lifecycle laboratory tests and mesocosm exposures have been used to assess the effects of chronic exposure to pulp and paper mill effluents: growth enhancement of invertebrates and fish, liver enlargement, and decreases in gonad size and fecundity in several fish species. Laboratory tests have assessed pulp and paper mill waste streams, and provided information to isolate and treat selected pulp and paper mill wastewaters. Long-term laboratory exposures of fish to pulp and paper mill effluents have examined reproductive parameters (egg production, time to first spawn). Effects on growth, maturation and reproduction have been seen in fish exposed to final effluents from a variety of pulp and paper mills, employing different pulping processes, and different bleaching chemicals.

The most sensitive endpoint in these lifecycle and long-term fish exposures is decreased reproduction. This endpoint is biologically meaningful, but determining thresholds for effects requires lengthy and expensive tests. As the laboratory tests move forward into the next decade, attention will focus on the reproductive endpoints, and the possibility of shortening the fish bioassays while still maintaining sensitivity and biological relevance.

Over the last decade laboratory bioassay development has been primarily driven by questions arising from field studies. Bioassay development has been critical for the confirmation of field effects under controlled conditions, to discriminate between historical and present-day discharges, to separate confounding stressors and as a short-term predictor of potential impacts on wild fish. Development of short-term bioassays, particularly in vitro tests mechanistically linked to whole organism responses, has facilitated investigations into the characteristics of chemicals associated with these effects.

Characterization of Bioactive Chemicals

A large body of work has been conducted in the last decade on investigating the sources and identities of bioactive substances present in final effluents from pulp and paper mills in Canada. Research has progressed in this area on two different fronts: questions regarding chemicals causing induction of detoxification enzymes (measured as ethoxyresorufin-O-deethylase or EROD activity) and chemicals causing reproductive effects in fish.

It was apparent in the early 1990s that there may be more than a single chemical or group of chemicals responsible for the impacts observed in wild fish. There was also a great deal of uncertainty about the role of chlorine bleaching and dioxins in the responses. By the mid 1990s it had been determined that dioxins and furans were associated with a portion of the induction of EROD activity however a significant fraction was attributed to unidentified compounds. It was also determined that effects were not correlated with organochlorine contents of effluents, as measured as adsorbable organic halogen (AOX). Research in the mid to late 1990s began to characterize chemicals associated with EROD induction. Several studies were able to partially attribute induction in fish to natural wood components liberated during the digestion of pulp or modified during bleaching processes. Other researchers working with wood extractives found that individually selected chemicals had the potential to affect fish reproduction but a lack of correlation between threshold responses and effluent concentrations indicated multiple compounds and mechanisms were involved. In the late 1990s research expanded into the identification of individual waste stream sources of bioactive substances. This, in concert with the advent of mechanistically linked *in vitro* and *in vivo* bioassays to drive investigative studies began to shed new light on the characteristics of chemicals affecting fish reproduction. There now appear to be multiple compounds affecting sex steroid signaling and gonadal production of sex steroids in fish. These compounds span a range of water solubilities, are readily bioavailable and accumulated rapidly by fish. This information is consistent with the body of evidence that has shown a sustained exposure is required to cause both elevated EROD activity and depressions in sex steroid levels. Collectively, these findings suggest that the responsible compounds are not persistent and do not bioaccumulate. The patterns of these substances in effluents and fish tissues are not correlated with a particular production type or effluent treatment strategy. There are also indications that compounds with similar activity can be extracted from wood.

From the work conducted to date there is strong evidence that bioactive substances originate from lignin. These compounds are derived from the original digestion of wood furnish and in kraft mills are present in black liquor, condensates derived from chemical recovery and black liquor carryover to the bleach plant. Additional bioactive substances are also present in bleach plant effluents that contain residual lignin removed during bleaching. The lack of a definitive identification of the responsible compounds has prevented an evaluation of the effectiveness of process changes synonymous to the pulp and paper industry. Continued research into the identities, origins and environmental fate of these substances and the efficacy of effluent treatment is required to determine their significance and relationship to the existing impacts of effluents from pulp and paper mills in Canadian aquatic ecosystems.

2. General Introduction

There are currently 134 pulp and paper mills in Canada (Lowell et al., 2003), and the industry employs over one million Canadians. Canada is one of the world leaders in the export of newsprint and market pulp (www.cppa.ca). The industry ranks second to domestic sewage in wastewater output to the Canadian environment. The pulp and paper industry employs a wide range of process types, up to 30 (Lowell et al., 2003).

Pulp is produced from raw wood material and is the basic ingredient in the production of paper. Wood consists of cellulose, hemicellulose, lignin and wood extractives (McLeay & Associates, 1987). The objective of pulping is to separate and recover cellulose fibers from lignin and other wood constituents with maximum yield and minimum fiber degradation. Pulping is carried out by the application of chemical or mechanical energy to wood. Kraft pulping is the most common process employed globally, where wood chips are cooked with sodium hydroxide and sodium sulfide to dissolve lignin. Other pulping processes involving the use of chemicals include soda and sulfite mills. Mechanical pulping involves shredding or grinding wood chips and usually results in high yield pulps; that is, a very high percentage of the original wood components are retained in the final product. Mechanical pulps are most commonly used for newsprint and catalogue paper (Biermann, 1996). Finally, semi-chemical pulping combines chemical and mechanical methods, where wood chips are partially digested in a weak chemical solution followed by mechanical refining for fibre separation (USEPA, 1998). Semi-chemical pulp is commonly used for manufacturing corrugated cardboard. After cooking in the kraft process, the pulp is washed and separated from residual cooking chemicals, known as weak black liquor. At bleached kraft mills, pulp is sent to a bleach plant where residual lignin is removed to achieve a desired brightness in the finished product; this is typically accomplished using a combination of chlorine dioxide and caustic extraction.

Large volumes of water are used in pulp production, which in turn generate significant effluent discharges to aquatic environments. A typical kraft mill discharges between 80,000 and 130,000 m^3/ day of effluent into surface waters. Effluents are a complex combination of waste streams produced in debarking, pulp washing, bleaching, and regeneration of cooking chemicals. Combined effluents are treated prior to release, typically in two stages. In primary treatment, suspended solids are removed in clarifiers and/or settling basins. In secondary treatment, microorganisms break down biodegradable material, which significantly improves effluent quality by reducing biochemical oxygen demand (BOD) and reducing levels of organic compounds associated with toxicity (Biermann, 1996; McLeay & Associates, 1987).

Environmental effects in the receiving waters downstream of pulp and paper mills, parallels the changes in the level of effluent treatment. From the 1950's to late 1970's, discharge of effluents high in fiber and BOD resulted in habitat degradation (*e.g.,* smothering of spawning beds due to fiber deposition, reduced oxygen concentration in the water column) and acute lethality to fish in receiving waters (McLeay and Associates, 1987; Folke, 1996; Owens, 1996). In response to these effects, regulators established "end-of-pipe" effluent quality limits for BOD and total suspended solids (TSS), an indication of the fiber concentration in effluents (Owens, 1991; Folke, 1996). Consequently, it was mandated that final effluents not be acutely lethal (*i.e.,* cause mortality to 50% of a test population) to standard bioassay species prior to release. The pulp and paper industry in North America responded to observations of environmental impact and regulatory concern by significantly improving effluent quality with better process and spill control and installation of effluent treatment systems (Smook, 1994; Folke, 1996).

In the 1970's and early 1980's, the focus of pulp and paper effects assessment was on the identification of the chemicals associated with the acute toxicity of final effluents (Owens, 1991, 1996; Folke, 1996). The toxicity associated with resin and fatty acids and chlorinated phenolics received much attention (Holmbom & Lehtinen, 1980; Kringstad & Lindström, 1984; Owens, 1991). In the late 1980's, pulp and paper production worldwide became an area of increased environmental scrutiny by the public as dioxins and furans were found in effluents and paper products.

In the 1980s discharges from Canadian mills were controlled federally by *Pulp and Paper Effluent Regulations* (PPER), which had been passed under the *Fisheries Act* (FA) in 1971. These set daily and monthly mass-based limits for BOD and TSS as well as the requirement that effluents not be acutely lethal to rainbow trout. The regulations did not address dioxin and organochlorine discharges. The regulatory limits were also only legally binding on mills constructed after the promulgation of the legislation in November 1971 which represented fewer than 10 percent of the mills in Canada. In order to address the deficiencies in the 1971 Regulations, the Minister of the Environment announced plans to revise the federal regulatory framework in March 1989.

At the same time, studies conducted in Sweden during the early 1980's provided some of the first evidence that effluents from some pulp mills were capable of inducing toxic responses in fish at very low concentrations in the receiving environment (reviewed in Södergren, 1992). The changes in growth, biochemistry and deformities in fish were dose-dependent and the area over which the effluents exerted health effects was considered to be large (> 8-10 km from the pulp mill; dilution of the effluent by more than 1000 times). Additional studies were also conducted at an unbleached kraft mill and relative to the bleached kraft mill, only small effects were demonstrated. Södergren (1989) regarded this as evidence of a relationship between the bleaching process and subsequent effects in the receiving environment. These conclusions were cited as the basis for using adsorbable organic halogens (AOX) to regulate pulp mills in Denmark, Sweden and Finland (Folke et al., 1991). These studies were widely and heavily criticized in several reviews because of the absence of supporting data from North American receiving waters (Sprague & Colody, 1989; Owens, 1991). The reviews suggested that the study site was unique and that such effects would not occur near well-operated mills. This assumption proved to be false, as studies conducted at Jackfish Bay on Lake Superior during the late 1980s found that fish exposed to primary-treated effluent from a bleached kraft pulp mill demonstrated very similar reproductive effects to those documented in Sweden. Fish exposed to effluent exhibited an increased age to sexual maturation, reduced gonadal development, reduced expression of secondary sexual characteristics and corresponding reductions in circulating reproductive steroid hormone levels (McMaster et al., 1991; Munkittrick et al., 1991).

In 1989-1990 the Canadian Federal government responded to the presence of dioxins and furans in effluents by conducting an assessment of effluents from mills using chlorine bleaching under the *Canadian Environmental Protection Act* (CEPA). The assessment concluded that bleached kraft mill effluent met the toxicity criteria used under CEPA. The assessment also concluded that the quality of the underlying science was insufficient to design regulations curtailing organochlorine discharges beyond what would be attained with the proposed dioxin-furan measures and new *Fisheries Act* regulations. Information deficiencies included knowledge of the make up of the organochlorine content of effluents and at that time it was not known which compounds were responsible for the observed environmental effects.

Federally, new regulations were passed under CEPA in 1992 to control releases of dioxins and furans. The existing PPER under the FA was updated and set stricter limits for BOD and TSS, while maintaining a similar non-acute lethality requirement as in the 1971 regulations. The limits in these new regulations became legally binding on all mills, unlike the earlier requirements. Very importantly, the new regulations included requirements for Environmental Effects Monitoring (EEM) at all mill sites. This allowed the effectiveness of the control limits in protecting fish, fish habitat and man's use of fisheries resources to be assessed. Compliance with the CEPA regulations entailed a capital investment of approximately $600 million for the affected 42 mills. The regulatory limits came into effect on passage in 1992, although mills which needed time for process modifications were allowed to defer compliance to January 1, 1994. The CEPA regulations on dioxins and furans were achieved by reducing the amount of dioxin precursors introduced to the process, as well as reducing the opportunity for the precursors to become chlorinated (Haliburton & Maddison, 2003).

Compliance with the new FA regulations entailed major changes in the way effluents were treated by the industry, requiring in most cases the installation of secondary treatment. In 1992, total capital costs of $2.3 billion were estimated for the 124 mills subject to these provisions (Haliburton & Maddison, 2003). For many mills, the prompt installation of controls was impeded by the poor market conditions of the time. The regulations therefore included provisions to allow eligible mills to defer compliance with limits to dates up to January 1, 1996, as justified by applications. Of the 124 mills subject to the regulations in 1996, 79 mills deferred compliance to then.

A retrospective report on the impact of the 1992 Federal Pulp and Paper Regulatory Framework on effluent quality was prepared. It demonstrates that dioxin and furan discharges have decreased more than 99%, biological oxygen demand of the effluents have decreased by 94% and total suspended solids by 70% (Halliburton & Maddison, 2003). Compliance rates with the new limits set under the 1992 regulations were also very high with mills meeting the BOD and TSS limits 99.8 % of the time and the rainbow trout acute lethality test 94.9% of the time (Halliburton & Maddison, 2003).

Under the FA regulations mills were also required to conduct EEM on a cyclical basis. EEM is a continuous, site-specific oriented program, aimed at providing a more complete and comprehensive understanding of the long-term impact of effluents on the Canadian aquatic environment. Research was a key activity to ensure continuous improvement in the scientific aspects of EEM. The constant evolution of the program was designed to allow mills to identify and differentiate causes of the observed effects, and to evaluate their significance, so that they may implement the necessary mitigation measures. To date, two cycles of studies have been completed and interpretive reports submitted to the National EEM office. Studies evaluate four areas:

- Sub-lethal toxicity testing, on 6-month intervals, to assess effluent quality;
- A benthic invertebrate community survey assessing fish habitat;
- A fish population survey to assess the health of fish; and
- A study of dioxin and furan levels in edible fish tissue for mills using chlorine bleaching, and a tainting study evaluating the usability of fisheries resources.

The cycle 1 reports were submitted in 1996, and covered sampling years from 1992 to 1996. Since about two-thirds of the mills sampled had yet to complete and bring on line secondary treatment plants in this period, sampling results in most cases represented the condition of biota exposed to primary treated effluents. The cycle 2 reports were submitted in April 2000 and covered sampling in the years 1997 to 1999. The cycle 2 results represented the biota's post-regulatory condition. The National EEM Office of Environment Canada and research scientists from the National Water Research Institute of Environment Canada have recently completed an assessment of the cycle 2 data (Lowell et al., 2003).

The CEPA assessment of bleached kraft mill effluents in the early 1990's observed that the quality of the underlying science was insufficient to design regulations curtailing organochlorine discharges beyond what would be attained with the new regulations. In order to fill the information gaps, in 1992 the Minister of the Environment launched an intensive government, industry and university research program. The original objectives of the research program were to 1) identify the causative organochlorine compounds, 2) determine how to eliminate them from the process, and 3) determine the short-term and long-term environmental effects of these compounds. Results from this research program along with feedback from the Environmental Effects Monitoring program would then be used to define what additional control actions may be necessary. The following document describes the research conducted from 1992-2002, following the implementation of the 1992 regulatory package. It is separated into three specific sections of research, 1) Field Studies and Mechanistic Research, 2) Development and Application of Bioassays, and 3) Characterization of Bioactive Chemicals.

3. Field Studies and Mechanistic Research

3.1 Summary

Studies conducted in Sweden in the early 1980s provided some of the first evidence that effluents from some pulp mills were capable of inducing toxic responses in fish at very low concentrations in the receiving environment In response to these findings, studies were initated in Canada and impacts of primary treated bleached kraft mill effluent on reproductive function in fish were found at Jackfish Bay, on Lake Superior. Additional studies confirmed these Swedish results at other bleached kraft mills in Canada. Research studies then determined that reproductive impacts in fish were not limited to mills that used chlorine in the bleaching process and they were also evident at some mills that employed secondary effluent treatment. Detailed mechanistic studies identified alterations in gonadal steroid biosynthetic capacity as the major site of reproductive action. These alterations in steroid biosynthesis lead to reductions in circulating levels of steroids and a cascade of other reproductive changes. Following the implementation of the new Pulp and Paper Effluent Regulations in 1992, studies continued at a number of locations and some signs of recovery were demonstrated following both process and treatment changes. The specific role of the individual process changes and the installation of secondary treatment on reproductive recovery however was unknown. A great deal of emphasis was placed on the difficulties in interpreting results from these kind of field studies and a number of collaborative research projects were developed to address these concerns.

As part of the new regulatory framework, an Environmental Effects Monitoring program was initiated which required mills to conduct receiving water fish and benthic invertebrate studies. Following the second cycle of the program, a national assessment was conducted identifying national response patterns in both benthos and fish endpoints. The finding of an average national response pattern consistent with the early studies at Jackfish Bay suggested that there were still concerns regarding pulp and paper mill effluent effects on reproduction in fish. At the same time, the measured increase in energy use and energy storage suggested that there were sufficient food for the fish, consistent with the eutrophication responses evident in the invertebrate studies. Over the last ten years we have made great progress in the area of field studies, first demonstrating reproductive effects, then determining the mechanisms of reproductive action, followed by the demonstration of partial reproductive recovery following process and treatment changes. It is clear however that mill effluents still impact the local receiving environments at a number of locations across Canada and that continued research involving bioassay application and chemical identification are required.

3.2 Canadian Research Leading Up to the 1992 Pulp and Paper Regulatory Package

In response to the results from the Swedish studies in the early 1980s, the first similar studies in Canada were conducted at a large bleached kraft pulp mill located in Terrace Bay, Ontario, which had discharged effluent into Jackfish Bay on Lake Superior since the late 1940s (Table 1, Figure 1). This research showed that white sucker (*Catostomus commersoni*) living in areas exposed to primary-treated effluent exhibited an increased age to maturity, and reduced gonadal development. In addition, male fish showed reduced expression of secondary sex characteristics and females showed reduced fecundity with age (McMaster et al., 1991; Munkittrick et al., 1991). These studies also demonstrated depressions in circulating levels of the major reproductive hormones and represented some of the first indications that some pulp mill effluents could disrupt hormonal systems in fish. These studies confirmed the previous Swedish work that had demonstrated that low levels of bleached kraft mill effluent affect fish growth, reproduction, and physiology (Södergren, 1989).

The pulp mill at Terrace Bay initiated secondary treatment of their effluent in October of 1989 in response to additional provincial regulations to address concerns over the acute lethality of the effluent. There were many improvements in effluent quality in response to secondary treatment, with reductions in BOD (92%), suspended solids (31%), chlorinated organics (AOX) to non-detect levels of dioxins and acute toxicity (35% LC50 to 100%) (Karl, 1992). Studies conducted in the fall of 1990 documented that the induction of hepatic mixed funtion oxygenase enzymes and reductions in circulating steroid hormone levels were not alleviated in fish following the implementation of secondary effluent treatment (Munkittrick et al., 1992a). This was not surprising since it was widely assumed that persistent organochlorines present in pulp mill effluents, such as dioxins and furans, were responsible for the physiological changes seen in wild fish near pulp mills (Rogers et al., 1989; Muir et al., 1990). However, these biochemical responses showed a marked improvement when fish were sampled after a short maintenance shutdown and reduced effluent flow to the receiving environment (Munkittrick et al., 1992b). These results suggested that 1) the compounds responsible for the biochemical changes were present in the secondary treated effluent, 2) contaminated sediments were not a large contributor to the responses seen at Jackfish Bay, 3) the chemical impacts were short-lived, and 4) if the responsible compounds were identified and removed, that recovery of fish populations might take place quickly (Munkittrick et al., 1997a, 1998). Additional studies at this location demonstrated similar physiological changes in hepatic mixed function oxygenases (MFOs) and steroids in lake whitefish (*Coregonus clupeaformis*) (Munkittrick et al., 1992a) and longnose sucker (*Catostomus catostomus*) (Munkittrick et al., 1992b) populations, although there were no consistent effects on whole-organism integrators between species. Lake whitefish showed a dramatic delay in maturity and reductions in gonad size (Munkittrick et al., 1992a), whereas white sucker showed moderate impacts (McMaster et al., 1991, Munkittrick et al., 1991), and longnose sucker showed few changes (Munkittrick et al., 1992b).

Figure 1. Timeline and evolution of research questions related to field studies of environmental impacts of pulp and paper effluents in Canada.

1982-1985	Swedish "Environment Cellulose" project identifies potential impacts near discharges
1988-1989	Are similar responses present in wild fish in Canada? (Initial studies at Jackfish Bay, ON)
1989-1990	Are similar effects present at other BKME sites in Canada? (La Tuque, Espanola)
1991	Do mills that don't use chlorine and that have secondary treatment cause effects in fish?
1992	Introduction of the new PPER and initiation of Research Program, EEM cycle 1 begins
1993	What are the consequences of inconsistent sampling stress?
1994	At mills that don't show effects, does effluent have the potential to cause effects?
1992-1996	What are the mechanisms responsible for the reproductive effects?
1993-1997	Do fish populations recover following the installation of secondary treatment and the various process changes?
1996-2000	Collaborative research studies designed to address a number of questions related to success of field studies; reference site and natural variability, confounding factors, use of forage fish etc.
1999-2002	Are reproductive endocrine effects present at the newest most modern mills in the country?
2002	What are the national response patterns in benthic invertebrates and fish from the cycle 2 EEM studies?

Table 1: Summary of Canadian studies of pulp and paper mill effects on fish. (Adapted from Munkittrick, 2003)

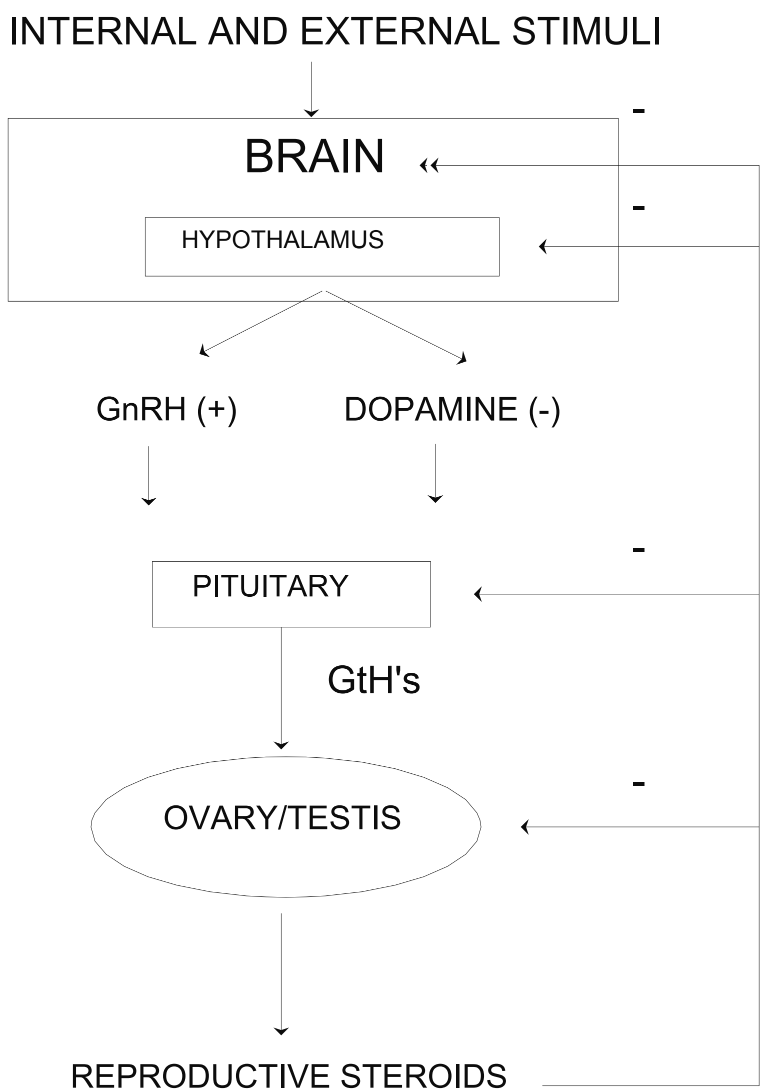
Location	Species		Mill type	Source or Reviewed in
1. Jackfish Bay, ON	White sucker	*Catostomus commersoni*	Bleached kraft	Munkittrick et al., 1998
	Longnose sucker	*Catostomus catostomus*		Munkittrick et al., 1998
	Lake whitefish	*Coregonus clupeaformis*		
2. Ontario (8+ mills)	White sucker	*Catostomus commersoni*	Various	Munkittrick et al., 1998
3. St. Maurice River, QC	White sucker		Bleached kraft	Bussières et al., 1998
4. Moose River basin	White sucker		Various	Munkittrick et al., 2000a Janz et al., 2001
	Trout perch	*Percopsis omiscomaycus*	Various	Gibbons et al., 1998b
5. Wapiti/Smoky River, AL	Longnose sucker	*Catostomus catostomus*	Bleached kraft	Swanson, 1994
	Mountain whitefish	*Prosopium williamsoni*		
	Longnose sucker Trout-perch	*Percopsis omiscomaycus*		McMaster et al., 2000
6. Athabasca River, AL	Spoonhead sculpin	*Cottus ricei*	Bleached kraft	Gibbons et al., 1998a
7. Winnipeg River, MN	White sucker	*Catostomus commersoni*	Groundwood/sulphite	Bezte et al., 1997
8. St. Francois River, QC	White sucker	*Catostomus commersoni*	Various	Kovacs et al., 2002
	Smallmouth bass	*Micropterus dolomieu*		
	Tesselated darter	*Etheostoma olmstedi*		
9. Fraser River	Chinook salmon	*Oncorhynchus tshawytscha*	Various	Wilson et al., 2000
10. Miramichi River, NB	Mummichog	*Fundulus heteroclitus*	Various	LeBlanc et al., 1997 Fournier et al., 1998
	Tomcod	*Microgadus tomcod*		Williams et al., 1998
11. New Brunswick and Nova Scotia (4 mills)	Tomcod	*Microgadus tomcod*	Various	Couillard et al. 1999
	White sucker	*Catostomus commersoni*		Galloway et al., 2003
12. Port Harmon, Birchy Cove, NF	Winter flounder	*Pleuronectes americanus*	TMP	Khan and Hooper, 2000 Khan and Payne, 2002
13. Ottawa River	Yellow perch	*Perca fluviatilis*	Various	McMaster et al., 2002
	Johnny darter	*1.1.1.1 Etheostoma nigrum*		McMaster et al., 2000 McMaster and Munkittrick 2000
14. Wapiti and Athabasca	Longnose sucker	*Catostomus catostomus*	Various	McMaster et al., 2003
	Trout perch Longnose dace	*Percopsis omiscomaycus*		
15 Various (65 mills)	Various		Various	Environment Canada, 1997 Munkittrick et al., 2002a Courtenay et al., 2002

Following identification of reproductive alterations in wild fish downstream of the bleached kraft mill located in Terrace Bay, Ontario, a number of studies were conducted in attempts to identify the mechanisms behind the reproductive alterations. White sucker of both sexes had reduced levels of the dominant sex steroid hormones throughout the period of gonadal development (McMaster et al., 1991). More in depth reproductive assessments were conducted during their spawning migration and identified a number of sites within the reproductive-endocrine axis affected by exposure to bleached kraft mill effluent (BKME), (Van Der Kraak et al., 1992) (Figure 2). Impacts included altered pituitary function (reduced circulating levels of gonadotropin-II (GTH-II) and diminished GTH-II response to a superactive gonadotropin-releasing hormone analog), reduced ovarian steroid biosynthetic capacity, and altered peripheral steroid metabolism. Despite these reproductive responses, exposure to BKME did not affect fertility of the eggs or sperm obtained from fish reaching sexual maturity nor subsequent larval development (McMaster et al., 1992). Continued studies at this site identified alterations of steroid productive capacity as one of the dominant alterations in reproductive function (McMaster, 1995) (Figure 2).

At the time of the early Jackfish Bay studies, a parallel effort was underway at other sites to determine whether the findings from the Swedish studies were seen near other North American bleached kraft pulp mills (Figure 1). Similar to the original Jackfish Bay study, one study examined fish downstream of an older technology pulp mill on the St. Maurice River, Quebec with high AOX and primary waste treatment (Table 1) (Hodson et al., 1992). Studies on the St. Maurice River found changes in liver glycogen levels, serum glucose, serum protein, and liver MFO levels that correlated well with tissue levels of dioxins and furans (Hodson et al., 1992). Additional studies at this site also demonstrated alterations in circulating levels of some of the major biologically active reproductive steroids (Gagnon et al., 1994a,b). Parallel studies were also conducted at a pulp mill on the Spanish River, Ontario with modernized processes and secondary waste treatment with a low AOX discharge (Table 1) (Servos et al., 1992). The collections at the modernized mill showed less dramatic responses, as fish downstream of the discharge demonstrated increased EROD activity and increased liver size, but there were no consistent reproductive effects of the effluent (Servos et al., 1992). Although AOX in the effluent discharges were quite different, due to the low dilution at the moderinized site, AOX in the receiving environments were quite similar (0.15 mg/L). With this said, the composition of the AOX was suspected to be quite different (Servos et al., 1992) with much lower levels of dioxins and furans expected at the moderinized site. Both studies however demonstrated some effects that persisted downstream, up to 51 km (Servos et al., 1992) and 95 km (Hodson et al., 1992) respectively. Whether effluent from modernized mills with secondary effluent treatment did not produce or release compounds capable of reproductive impacts was not known.

Another series of studies were initiated at a bleached kraft mill at Grande Prairie on the Wapiti River, Alberta that was relatively new (1973) and that had secondary effluent treatment for the entire life of the mill (Table 1; Kloepper-Sams et al., 1991; Kloepper-Sams & Swanson, 1992). These studies found no evidence of whole organism changes in either longnose sucker or mountain whitefish, and few consistent changes in a large number of physiological parameters. The absence of significant changes in physiological parameters in the Wapiti River studies could have been related to a number of factors, including the pulping process and waste treatment employed by the mill, the dramatic seasonal changes in dilution, or the high mobility of the fish species present (Swanson et al., 1993, 1994; Kloepper-Sams & Benton, 1994; Kloepper-Sams et al., 1994; Owens et al., 1994a,b; Pryke et al., 1995).

Figure 2. Schematic representation of the control of steroid production by the hypothalmo-pituitary-gonadal axis.



The results of the early Jackfish Bay studies also prompted a survey of other large Ontario pulp and paper mills, to determine whether similar responses were evident in wild fish downstream of mills that utilized different processes (Table 1)(Robinson et al., 1994). All mills selected discharged more than 40,000 m^3 of effluent per day and represented a range in available process and effluent treatment technologies for the time of the study (Robinson et al., 1994). The main objectives of the study were to determine whether the discharge of effluent from pulp and paper mills to sites other than Jackfish Bay was associated with physiological or biochemical disruptions in wild fish, whether there was any correlation between waste treatment and the presence of biological responses in wild fish, and whether there was any association between the use of chlorine as a bleaching agent and these responses (Figure 1)(Munkittrick et al., 1994a). The survey also examined the relationships between responses seen in wild fish with toxicity tests conducted on receiving water samples (Robinson et al., 1994), measurements of liver and muscle levels of dioxins and furans in white sucker (Servos et al., 1994) and the correlation of dioxin-level estimates in white sucker liver derived from the rat H4IIE assay (van den Heuvel et al., 1994).

The endpoints measured in wild fish included whole body measures (length, weight, condition factor and age), organ weights (liver and gonad) as well as hepatic ethoxyresorufin-o-deethylase (EROD) induction and measurement of circulating steroid hormone levels. Preliminary examination of the whole body measures indicated that alterations were evident in fish collected downstream of all effluent discharges, although responses were not consistent at all sites (Munkittrick et al., 1994a). The absence of chlorine bleaching or the presence of secondary treatment did not appear to eliminate responses in fish, including decreased gonadal size and increased liver size.

3.2.1 1st International Conference on the Environmental Fate and Effects of Bleached Pulp Mill Effluents

As pulp and paper production worldwide had become an area of increased environmental scrutiny, and the original Swedish studies and the new Canadian studies were being discounted (Owens, 1991), the Swedish Environmental Protection Agency organized and held the 1st International Conference on the Environmental Fate and Effects of Bleached Pulp Mill Effluents, November 19-21, 1991 in Stockholm, Sweden. The recent Canadian findings were very prominent at this conference as Dr. Kelly Munkittrick opened the conference with the plenary lecture by stating that “Virtually all findings in the early Scandinavian investigations (1982-1985) of the impact of bleached kraft pulp mill effluent (BKME) on fish, including effects on mixed function oxygenases (MFO), conjugation enzymes, energetics, haematology, immune responses, non-specific biomarkers, growth and reproduction, have been found in North America, but changes are inconsistent and vary from site to site “(Munkittrick, 1992). Thus at this time, there was consensus that similar reproductive disturbances appeared in the receiving environment of bleached kraft pulp mills in other parts of the world, but there was no clear solid evidence of which compounds were the culprits. Although organochlorines were still thought by some, to be the main suspects (Södergren, 1996), there was preliminary evidence that they may not be the only compounds responsible (Munkittrick et al., 1994a). Within Canada, some of the questions that remained were whether 1) changes were found at mills which do not employ chlorine bleaching, 2) what treatment strategies were capable of removing the biologically active components and, 3) what was the relationship, if any, was there between induced MFO enzymes and the concurrent disruptions in gonadal steroid metabolism (Munkittrick, 1992).

Following this international conference, in December of 1991, the Canadian Federal Government announced a Pulp and Paper Regulatory Package that included regulations on the discharge of BOD, suspended solids and acute toxicity for all pulp and paper mills, as well as placing strict limits on discharges of chlorinated dioxins from pulp mills employing bleaching (Figure 1). The Canadian pulp and paper industry then began to alter its processes and treatment strategies to meet these new regulations over the next number of years. At the same time, in order to fill some of these information gaps, the Minister of the Environment launched an intensive government, industry and university research program. The original objectives of the research program were 1) to identify the causative organochlorine compounds, 2) to determine how to eliminate them from the process, and 3) to determine the short-term and long-term environmental effects of these compounds (Figure 1).

3.3 Research Program to Identify the Causative Compounds, How to Eliminate Them, and Determine Their Short and Long-Term Environmental Effects

The results from the large survey of Ontario pulp and paper mills were completed and published. This study was designed to investigate the receiving areas below a large number of pulp and paper mills to determine whether discharges were associated with any of the physiological responses evident in fish collected in Jackfish Bay, Lake Superior. It was also designed to determine whether there was any correlation between waste treatment and the presence of biological responses in wild fish, and whether there was any association between the use of chlorine as a bleaching agent and these responses (Figure 1) (Munkittrick et al., 1994a).

Alterations were demonstrated in fish collected downstream of all effluent discharges, although responses were not consistent at all sites (Munkittrick et al., 1994a). Although white sucker collected near bleached kraft mills exhibited the highest EROD induction and dioxin levels (Servos et al., 1994), elevated enzyme activity was observed in fish from sites that did not use chlorine, and depressions in sex steroid levels were not correlated with the level of EROD activity (Munkittrick et al., 1994a). The absence of chlorine bleaching or the presence of secondary treatment did not eliminate responses in fish, including decreased circulating levels of sex steroids, decreased gonadal size and increased liver size (Munkittrick et al., 1994a). Although there was a positive correlation between EROD and dioxin equivalents overall, some discrepancies suggested that additional compounds may be involved (Servos et al., 1994). There were no relationships between reproductive responses in wild fish downstream of the mill sites and dioxin equivalents (Servos et al., 1994) or with any of the receiving water laboratory toxicity tests (Robinson et al., 1994). The surveys' main findings were that a) induction of hepatic EROD enzymes and depressions of some plasma sex steroids were found downstream of several pulp and paper mills; b) these changes were seen at some mills without chlorine bleaching and at mills that had secondary treatment; c) substantial dilutions of non toxic effluent did not appear to remove these responses; d) the dominant factor determining the presence or absence of responses appeared to be dilution level; and e) lab toxicity tests on invertebrates, rainbow trout, and fathead minnows could not predict the presence of these responses in wild fish (Munkittrick et al., 1994a). In conclusion, there was no correlation between reproductive effects in wild fish and pulping process, dioxin contamination, AOX production, AOX levels in the receiving water or the use of chlorine. The identity of the chemical(s) responsible for the reproductive effects remained unknown and it was not known whether the addition of chlorine to the chemical(s) altered their potencies.

Studies also continued at a number of the locations examined prior to the new effluent regulations. Servos et al., (1992) had previously documented minimal effects at a modernized bleached kraft mill on the Spanish River in Northeastern Ontario. This mill was also included in the larger survey of Ontario mills and although some steroid differences were demonstrated, impacts on gonadal development were not found (Munkittrick et al., 1994a). However, effluent from this mill was shown by Robinson (1994) to affect sexual maturation and hormone levels in fathead minnows during chronic laboratory exposures, although at concentrations above those found in the receiving environment (Figure 1). This information was important because it demonstrated that some modernized mills may produce or release compounds capable of reproductive impacts, but fish at a specific receiving environment may not show responses for any of a variety of reasons, including species sensitivity, effluent dilution or site-specific receiving environment conditions which may offer the fish refuge from exposures. It is important to determine whether the absence of responses in wild fish at any specific site, are because the mill does not produce the chemicals or the fish are protected by some other mechanism.

Follow-up studies were also conducted at the two sites on the Moose River basin that were included in the earlier 10 mill study (Table 1). Nickle et al. (1997) conducted more detailed studies at Smooth Rock Falls (BKME) and Kapuskasing (Thermo-mechanical pulp - TMP) prior to the installation of secondary treatment at these sites in order to provide detailed pre-secondary treatment data to potentially follow recovery. His studies confirmed both physical and biochemical changes in white sucker exposed to both primary treated BKME and TMP mill effluent. Both mill effluents contained compounds that induced MFO activity, increased peroxisomal fatty acyl-CoA oxidase activity and reduced circulating sex steroid levels. However, in this study, exposure to the BKME discharge was associated with reductions in gonadal development, whereas the TMP discharge had no effects on gonad size but led to increases in liver size. Earlier studies at the TMP site however, did demonstrate differences in ovarian development downstream of the TMP discharge (Munkittrick et al., 1994a). The reproductive alterations documented did not appear to be in response to estrogenic substances present in the effluent as no induction of vitellogenin was evident. In fact reductions in vitellogenin in females downstream of the primary treated BKME site corresponded to reductions in circulating 17ß-estradiol levels (Nickel et al., 1997). Increases in peroxisomal proliferation within the liver tissue may have been partially responsible for the increase in liver size in white sucker downstream of the primary treated TMP mill in Kapuskasing.

Studies on the St. Maurice River found changes in liver glycogen levels, plasma steroids, and liver MFO levels (Hodson et al., 1992; Gagnon et al., 1994a,b). Continued study at this site examined in more detail the reproductive alterations demonstrated in the original studies. These follow-up studies concluded that the depressions in steroid levels were not related to changes in hormone biosynthesis, but rather to increased metabolism (Gagnon et al., 1994b). Some of the differences between the conclusions of the Jackfish Bay and Moose River studies and those of Gagnon et al., (1994a,b) could have been due to the effects of capture and handling stress on steroid hormone levels (Van Der Kraak et al., 1992). Follow-up studies at Jackfish Bay demonstrated that handling stress (McMaster et al., 1994) and confinement stress (Jardine et al., 1996) led to dramatic changes in physiological parameters, and that holding fish for a short time period to “recover” was not sufficient for overcoming these effects of capture stress in the white sucker species (Jardine et al., 1996).

The 2½ year, multidisciplinary study of the Wapiti/Smoky River ecosystem in northwestern Alberta was also completed.

The Weyerhaeuser Canada Ltd. mill was one of the newest most modern mills in the country and during these studies upgraded their process from 25 to 70% chlorine dioxide substitution in the fall of 1990 and to 100% substitution in July of 1992. Overall findings were that AOX did not correlate with dioxins, furans, chlorinated phenolics or environmental impacts and that implementation of 70% chlorine dioxide substitution resulted in declines in dioxins/furans and chlorinated phenolics to near or below detection limits (Swanson et al., 1993). Although fish demonstrated induction of the liver detoxification system that paralleled dilution of the effluent, it was not correlated with health effects in mountain whitefish or longnose sucker. These fish did demonstrate a response related to nutrient enrichment which led to increased condition factor and increased internal fat stores (Swanson et al., 1993). The absence of significant changes in physiological parameters in the Wapiti River studies could have been related to a number of factors, including the pulping process and waste treatment employed by the mill, the dramatic seasonal changes in dilution, or the high mobility of the fish species present (Swanson et al., 1993, 1994; Kloepper-Sams & Benton, 1994; Owens et al., 1994a,b; Pryke et al., 1995).

Studies continued at the original Jackfish Bay location with a number of different objectives. Although numerous improvements had occurred in Jackfish Bay following the installation of secondary treatment (Karl, 1992), white sucker had not demonstrated similar improvements in reproductive function. For this reason, wild fish were continually monitored throughout the reproductive growing season for evidence of potential recovery following the installation of secondary treatment in the fall of 1989. The first signs of potential recovery in reproductive function were demonstrated in early September of 1993. Although gonadal size differences were still evident, no site differences in circulating steroid levels (Jardine, 1994) or *in vitro* production (McMaster unpubl. data) were found. However, circulating and *in vitro* differences were present in samples collected in late September and late October 1993, at this same site (McMaster et al., 1996a). The first signs of wild fish recovery corresponded well with caging studies conducted with goldfish, as studies conducted during the spring of 1993 demonstrated effluent potential to reduce steroid production, while studies conducted in the summer and fall of 1993 as well as the spring of 1994 failed to demonstrate reproductive effects using this short-term exposure protocol (McMaster et al., 1996a) (See *Development and Application of Bioassays*). This partial recovery in reproductive function at the time coincided with a series of mill process changes in May and June 1993 associated with increasing the level of chlorine dioxide substitution to 70% (Munkittrick et al., 1997a).

Early studies at Jackfish Bay concluded that one of the main factors responsible for lowering sex hormone levels during prespawning periods was occurring within the steroid biosynthetic pathway in a reduced ability to make steroid hormones (Van Der Kraak et al., 1992). McMaster et al. (1995a) continued these studies and determined that BKME exposed white sucker also have reduced steroid biosynthetic capacities during both early and later stages of vitellogenic growth. Further studies identified specific locations within the steroid biosynthetic pathway impacted, such as reduced aromatase activity during early vitellogenic stages and disruptions downstream of pregnenolone formation during later stages of vitellogenic growth (McMaster et al., 1995a). Continued studies into impacts in the biosynthetic pathway measured both the levels of the steroid precursor substrates and assessed the enzyme conversion rates within the steroid biosynthetic pathway. These studies identified specific lesions associated with this decreased steroid productivity and further showed that these particular sites of disruption within the pathway differed with the reproductive state of the fish. Although a number of locations within the steroid biosynthetic pathway were altered with BKME exposure, the major alteration appeared to be due to reductions in the availability of the steroid substrate cholesterol (McMaster, 1995; McMaster et al., 1996b).

Bezte et al. (1997) conducted a study on the Winnipeg River downstream of a primary treated groundwood/sulphite pulp and paper mill in Pine Falls, Manitoba (Table 1). Similar to some of the mills in the 10-mill survey, this mill also did not bleach pulp. White sucker collected downstream of the effluent discharge demonstrated induced EROD activity, altered fecundity, reduced hepatic vitamins, increased liver sizes, and reductions in circulating steroid levels (Bezte et al., 1997). Examination of the ability of ovarian follicles to produce reproductive steroids at this site also demonstrated a reduced capacity of the gonads to produce these reproductive steroids (McMaster et al., 1996b) similar to those documented at Jackfish Bay on Lake Superior (McMaster et al., 1995a).

3.4 Evolution of the Research Questions

This group of early Canadian research studies on the impacts of pulp and paper mill effluents on biota in the receiving environment answered a number of questions, but lead to the development of many new questions. It was now evident that reproductive alterations in wild fish in Canada were not unique to Jackfish Bay and that mills that did not use chlorine also demonstrated effects (Figure 1). Some studies at modern mills also failed to demonstrate changes in fish populations. The original objectives of the research program launched by the Minister were to identify the causative organochlorine compounds, to determine how to eliminate them from the process and to determine the short-term and long-term environmental effects of these compounds. The identity of the chemical(s) responsible for the biological activity remained unknown, but the presence of changes at nonchlorinated mills suggested that the chemical structure of the inducing compound(s) does not require chlorine for its biological activity. It was also not known whether chlorine bleaching resulted in chlorination of the biologically active compound(s) and, if so, whether the presence of chlorine bleaching affected the threshold or persistence of biological effects.

3.4.1 2nd International Conference on the Environmental Fate and Effects of Pulp and Paper Mill Effluents

In November 1994, over 280 scientists from more than a dozen countries gathered in Vancouver, British Columbia, Canada to examine and compare new developments regarding the fate and effects of pulp mill effluents (Servos, 1996). Environment Canada hosted the conference and together with the Department of Fisheries and Oceans produced a book of the peer reviewed conference papers. The fact that the title of the book changed from the fate and effects of pulp mill effluents to the fate and effects of pulp and paper mill effluents indicated one of the dominant findings of research since 1991. Anders Södergren (1996) stated that, “The last three years had been one of dramatic change in the pulp and paper industry. In 1991, words like elemental chlorine free (ECF), totally chlorine free (TCF) and totally effluent free (TEF) did not exist. Today they were common language in use in various forms in the industry”.

Of the close to 100 presentations at the 1st international conference in Sweden, the majority of the work (64%) was conducted on the chemistry aspect of pulp mill effluents. Of the biological studies presented, roughly 30% were large integrated assessments of the impacts of bleached kraft mill effluents on receiving environments. At the 1994 conference in Canada, approximately 100 presentations were presented, however there was a dramatic shift from more presentations on the chemistry of the effluents to equal numbers on chemistry and biology. Within the biology presentations, there was also a shift from the larger integrated assessments towards studies examining the biochemical responses of organisms towards effluent exposure.

In terms of advancement of the science from 1991 to 1994, in 1991 a number of questions were left unanswered as to the relevance of the consistent induction of MFO activity in fish downstream of effluent sources. Hodson (1996) identified the unanswered questions from 1991 and summarized where the research over the last 3 years had taken us;

a. While MFO induction of fish is often strongly associated with other responses to BKME, there are no defined mechanisms linking induction to specific effects.
b. The environmental fate of MFO inducers suggests two classes of compounds, those that persist (dioxins) and those that are labile (unidentified compounds).
c. Chlorine is not essential for MFO induction.
d. The role of chlorine bleaching in releasing inducers from pulp and in changing their potency through chlorination is unknown. AOX concentrations in effluents are unrelated to MFO induction potency.
e. Enhanced effluent treatment can reduce the potency of MFO induction.
f. Of the two classes of inducers, the persistent, chlorinated class was no longer a problem in mills that reduced dioxin emissions through process change, while labile inducers appeared readily metabolized by fish.
g. The toxicological and ecological relevance of MFO induction by pulp mill effluents cannot be determined without identifying the inducing compounds. It is imperative that inducers in effluent be isolated, purified and identified to answer these questions.

Sandström (1996) indicated that by 1994, it was more widely accepted that pulp mill effluents constitute a threat to fish populations in the receiving waters with the confirmation of the earlier Swedish results by the North American studies. Sandström (1996) summarized reported observations from 18 different mill locations in Sweden (6), Canada (11) and the United States (1), totaling 30 studies covering 8 different species. In all, of the studies that examined sexual maturation, 80% found that it was negatively affected, gonad size was depressed in almost 60% of the studies and condition factor was increased roughly 50% of the time. Of the mills that demonstrated no impacts, high dilution of the effluent in the receiving environment was common. Therefore, it appeared that fish downstream of effluent sources were receiving sufficient food supply, but were unable to use this energy resulting in impaired sexual performance (Sandström, 1996).

Sandström (1996) also referred to the recent adoption of new guidelines for environmental effects monitoring programs in Canada and Sweden. Both programs utilize whole organism-level measurements in one or two sentinel species to indicate changes in energy stores and reproductive performance as well as age structure of the population to indicate differences in recruitment patterns indicating reproduction failure. Implementation of these two programs was thought to be able to provide increased information as to the extent of impacts of pulp and paper mill effluents within these two countries.

Owens (1996) summarized the Canadian results by stating “In an overall regulatory context, the Environment Cellulose findings in conjunction with the discovery of TCDD/TCDF led to an almost exclusive focus on chlorinated organics and bleaching”. Several nations rapidly implemented or proposed AOX regulations, even though the scientific basis for using AOX as a regulatory parameter was questioned (Folke et al., 1991). The Canadian data was instumental in dispelling the association between effects and chlorinated organics. As a result, Canada declined use of AOX as a regulatory parameter. Canada has since proceeded to focus research to identify causative agents using parallel field work with chemical effluent characterization and laboratory exposures.

3.5 Evolution of the Research Questions: Monitoring Sites over the Long-term for Evidence of Recovery Following Process and Treatment Changes.

Although a number of Canadian studies had documented reproductive effects in wild fish downstream of effluent discharges, a number of these mills had only primary treatment of their effluent. Following the implementation of the new pulp and paper effluent regulations, mills started to install secondary treatment systems and mills that bleached their pulp were altering mill processes to reduce the use of elemental chlorine. We knew now that chlorine was not required for reproductive effects and that secondary treatment did not eliminate effects at some sites. As some modernized mills failed to demonstrate effects, mills that were being modernized were monitored for potential recovery in the receiving environment over the next several years (Figure 1).

Over the years of study at Jackfish Bay there had been multiple changes in processes and treatment strategies. There was corresponding evidence of reduced activity of the effluents and recovery in reproductive potential in wild fish populations. Steroid depressions seen in 1990-1993 were associated with effects on the steroid biosynthetic pathway (Van Der Kraak et al., 1992; McMaster, 1995; McMaster et al., 1995a, 1996b). These effects varied with season (McMaster, 1995; McMaster et al., 1995a) and were not consistent at all mills (McMaster et al., 1996b). Reductions in production of hormones *in vitro* could be seen following 4 to 8 days of exposure to effluent at Jackfish Bay until the summer of 1993 (McMaster et al., 1996a). In the summer of 1993, there was a partial recovery in the steroid impacts (McMaster et al., 1996a), and goldfish during short- term exposures did not show depressions in steroid levels for exposure periods as long as 20 d in 100% effluent (McMaster et al., 1996a; McCarthy et al., 1997). No specific in-plant change was identified which could account for the improved performance.

It was necessary to evaluate whether wild fish showed a response to the apparent changes in effluent treatment. Fish collected in Jackfish Bay during early September of 1993 showed a recovery in hormone levels (Munkittrick et al., 1997a), however differences were present again in samples collected during late September and late October 1993 at this site (McMaster et al., 1996a). Male gonad sizes were not significantly different in the fall of 1995 and although some improvements in MFO induction had been seen, levels in both sexes remained substantially elevated relative to reference fish (Munkittrick et al., 1997a). Although there was evidence of improvement in gonadal development in the fall of 1993 during early gonadal recrudescence (gonadal growth), white sucker collected immediately prior to spawning did not show as marked an improvement. Female white sucker did show an increase in median ovarian weight of more than 50 g relative to 1988 and 1989, but gonadosomatic indices were still significantly reduced relative to reference fish. Although circulating hormone levels in both males and females remained at low levels, there was an apparent recovery in secondary sex characteristics (Munkittrick et al., 1997a). In 1989 and 1990 males showed a marked reduction in the expression of secondary sex characteristics (McMaster et al., 1991), whereas in the spring of 1996 males showed similar or higher prevalence of tubercles relative to the reference site. However, some tubercle development was displayed in 50% of female fish, especially on their anal fin (Munkittrick et al., 1997a).

The partial reproductive recovery identified may also have been associated with changes in the biosynthetic pathway. Although the circulating levels of hormone had not recovered, there had been significant changes within the steroid biosynthetic pathway. Previous experiments demonstrated lesions at several places within the pathway (McMaster, 1995; McMaster et al., 1995a, 1996b) that converts cholesterol to the active steroid hormones. Although prespawning lesions were seen at the bottom of the pathway in 1992 and 1993 (McMaster, 1995; McMaster et al., 1996b), incubations conduced during prespawning in 1996 showed no disruptions within the pathway. It was unclear why circulating levels of the hormones remained depressed to levels similar to the early 1990s despite the apparent recovery of function within the pathway. The low basal *in vitro* production would appear to be related to cholesterol availability as opposed to previous disruptions within the steroid biosynthetic pathway.

It was possible that fish exposure in Jackfish Bay also changed with the installation of secondary treatment. The effluent temperature and oxygen levels changed substantially following treatment, potentially allowing fish to increase their exposure concentration to the responsible compounds. This may account for some of the continued reproductive effects at Jackfish Bay. The relationship between steroid hormone changes and reductions in gonadal development was still unclear.

Studies also continued in the Moose River Basin in Northeastern Ontario with a number of objectives. Research conducted in 1991 at Kapuskasing and Smooth Rock Falls had shown that fish downstream of these mills exhibited induction of liver detoxification enzymes and depressions of plasma sex steroid hormones during the period of gonadal growth (Munkittrick et al., 1994a). Subsequent studies were initialed to follow process changes and waste treatment installation at Kapuskasing (TMP) and Smooth Rock Falls (BKME) (Nickle et al., 1997; Munkittrick et al., 2000a). Both of these mills were situated on rivers that had a number of obstacles to prevent fish movement. Both mills were situated at a small run of the river dam and additional waterfalls were located downstream. These obstacles prevented fish from altering their exposure profiles following the installation of secondary treatment as they may have at Jackfish Bay. The Kapuskasing mill initiated changes in late 1991, and by the fall of 1993, both the magnefite and groundwood processes were switched over to TMP (Munkittrick et al., 2000a). White sucker collected in the fall of 1993 had similar gonad size but markedly increased liver size. The downstream site (Freddy Flats) was 12 to 14 kms below the mill outfall, and in 1994 a second downstream site was added within 2 km of the discharge. Fish from these sites showed much larger livers than the Freddy Flats fish and no reductions in gonad size (McMaster et al., 1997a,b; Munkittrick et al., 2000a).

Kapuskasing installed an activated sludge waste treatment system, which became operational in April 1995, after which liver size was reduced substantially but still remained larger than that at the reference sites. Condition factor also showed a gradual decline back to reference levels (Munkittrick et al., 2000a). At Smooth Rock Falls, ovarian sizes and hormone levels remain depressed despite 100% chlorine dioxide substitution as well as secondary waste treatment (Munkittrick et al., 1998). However, gonadal sizes recovered 40km downstream, when effluent concentrations had fallen to about 1.3% (McMaster et al., 1997b). Tightening the pulping process to reduce black liquor losses in 1997 may have contributed to the improvements in fish performance (Munkittrick et al., 2000a).

It was not known what process changes were responsible for reducing the reproductive responses seen at some locations. The best information was available from a series of studies conducted by Kovacs et al. (1995a,b; 1996) in which they conducted fathead minnow life cycle studies at a bleached kraft mill before and after a series of mill upgrades and process changes. The first studies demonstrated a number of reproductive effects, however no reproductive responses were demonstrated in the follow-up experiment. Mill process changes implemented included increasing ClO_2 substitution from 45 to 100%, improved brownstock washing, increased efficiency of the steam strippers, increased recycling of the foul condensates, changes in some sewer destinations, and increasing the retention time in the secondary effluent treatment system (Kovacs et al., 1996). This was also consistent with field collections, as wild spoonhead sculpin (*Cottus* ricei) downstream of the mill discharge after modernization, were larger than upstream fish and showed no decreases in reproductive parameters (Table 1)(Gibbons et al., 1996).

The Northern River Basins Study (NRBS) was established in 1991 and represented a joint federal, provincial and territorial agreement between the governments of Canada, Alberta and the Northwest Territories. The primary objective of the study was to advance the understanding of how developments, particularly the seven pulp mill developments, within the Peace, Athabasca and Slave river basins had cumulatively impacted the aquatic ecosystem. The study was also to provide the necessary knowledge base and tools required to assess the potential consequences of future pulp and paper mill developments in these basins. Fish health in the NRBS was assessed using a suite of biomarkers and measures of general condition. The weight of evidence indicated that many of the fish in these basins exhibited signs of physiological stress. Of particular concern was the finding that sex hormone levels in burbot and longnose sucker collected from near-field pulp mill locations were significantly depressed, and that numbers of immature fish in these same locations were unexpectedly high and were more likely to show signs of external abnormalities (Wrona et al., 1997). As with fish, the primary effects on benthic macroinvertebrates appeared to occur in the areas immediately downstream from pulp and paper mill and municipal sewage discharges. Measures of benthic macroinvertebrate community structure in the areas immediately above and below pulp and paper mills indicated that while communities did not differ in terms of major taxa, there was a tendency for downstream communities to show higher diversity and density (Wrona et al., 1997). This was most likely a consequence of nutrient enrichment, an observation supported by Chambers (1997), indicating that pulp and paper mills contributed approximately 13% of the annual total nitrogen and phosphorus loads in the Athabasca and Smoky-Wapiti Rivers.

3.5.1 Cycle 1 of the Environmental Effects Monitoring Program

In 1992, as part of the amendments to the Pulp and Paper Effluent Regulations (PPER), every mill was also required to conduct an Environmental Effects Monitoring (EEM) program. The objective of the EEM program was to determine if the fish, fish habitat and the utilization of the fisheries resources were protected by the requirements of the regulations. Given the results from previous research studies in Canada, this program would provide evidence as to how widespread impacts of pulp and paper mill effluents on the Canadian receiving environment were. The study components included: an adult fish survey; an invertebrate community survey; measurements for chlorinated dioxins and furans in fish tissue for mills using chlorine; and a fish tainting study if there were public concerns about this issue. It was developed as a tiered, site-specific, cyclical program, which would iteratively lead to conclusions concerning the presence of effects in the effluent discharge area, their geographic magnitude and extent, and the relationship of the effects to the discharge. As the pulp and paper program developed towards cycle 1 in 1993, there was considerable controversy about the design of the program and the potential interpretability of the data. This led to a change in the primary objective of the first cycle Adult Fish Survey to obtain estimates of fish variability in order to allow a proper statistical design in the next cycle of the program. After the first cycle of monitoring was completed in 1996, expert working groups were formed to review the results and to make recommendations to improve the requirements for the second cycle. They reviewed the successes, problems, variability, and suitability of methodology and summarized the data. These groups developed additional guidance and streamlined methodologies, and in many cases, revised guidance documents that were used in the development of Cycle 2 of the EEM program.

One of the primary components of the EEM program was the fish survey, which monitors the growth, reproduction and condition of fish populations in waters receiving mill effluents. Specific fish measurements reflect the energy use (growth rate, gonad weight, fecundity, age at maturity), energy storage (condition, liver weight) and age (mean age or age distribution) of two sentinel fish species (Munkittrick et al., 2002a). The objective of the first cycle of EEM was to collect baseline information (fish species selected, capture techniques, sampling areas) and to determine variability in fish measurements. In Cycle 1, the fish survey was poorly conducted at many mills. The key issues identified by the fish survey expert working group (FSEWG) included: the inability to catch sufficient numbers of fish, uncertain exposure, poor reference site selection, the presence of confounding factors and poor or incomplete reporting of the data (Fish Survey Expert Working Group, 1997). The FSEWG recommended a number of improvements to the fish survey including: the selection of appropriate sentinel species such as small-bodied (presumably more sedentary) fish, study design and sampling to ensure the required numbers of fish were collected. As well, a decision tree was developed to help assess whether the fish survey or an alternative was appropriate at any specific site (Fish Survey Expert Working Group, 1997).

The objective of the invertebrate community survey was to delineate the magnitude and spatial extent of habitat degradation due to organic enrichment or other forms of contamination (Environment Canada, 1998a). The intent of the first cycle of EEM was to characterize the benthic invertebrate communities in major habitats that may be affected by mill effluent and to establish a baseline against which future cycles could be compared. Results from Cycle 1 indicated that 69% of pulp and paper mills found differences between reference and exposure sites for the endpoints of species richness and abundance (Walker et al., 2002). However, data interpretation was often confounded by factors such as multiple effluents, inappropriate reference site selection, and lack of standardized methodology for sampling and data analysis (Benthic Invertebrate Community Survey Expert Working Group, 1997). Similar to the Fish Survey EWG, the Benthic Invertebrate Community Survey EWG made a number of recommendations to address these problems, which were then incorporated into the Technical Guidance Documents (Environment Canada, 1998a).

3.5.2 3rd International Conference on the Environmental Fate and Effects of Pulp and Paper Mill Effluents

The third international conference on the Environmental Fate and Effects of pulp and paper mill effluents was held in Rotorua, New Zealand from November 9-13, 1997. Munkittrick and Sandström (1997) examined the proceedings from the first two conferences and compared how the studies and study designs had changed over the last 6+ years. Since the 1991 conference, there had been a marked increase in the number of conference presentations documenting effects of process changes, a direct result of massive investments by industry in new processes and waste treatments to deal with changing regulations specifically in Canada and Sweden. There had not been however, corresponding increases in large ecological studies examining effects in the receiving environment or improvements following process changes. At the conference, there was also considerable controversy about several aspects, including the range of perspectives from, 1) there was no issue, versus 2) the issue was solved, versus 3) more work was still needed. As well, there was also considerable controversy regarding the role of ecological field studies and their usefulness (Munkittrick & Sandström, 1997).

3.6 Evolution of the Research Questions: Need to Identify Process and Treatment Changes Responsible for Partial Recovery and Chemicals Involved

Partial recovery had been demonstrated at a number of locations within Canada, although the specific process or treatment changes responsible had not been identified. There remained the need to identify these process and/or treatment changes. It was also clear from both field and laboratory studies that an increased age to maturity appeared to be a more sensitive response than egg quality or spawning. Therefore, there was also a need to further identify the mechanisms responsible for the reproductive impacts and to evaluate which factors controlled the initial growth and maturation of gonads in immature fish. There was also the need to identify the chemicals responsible for the physiological impacts in order to design and implement the process changes to remove them (Figure 1). As questions about the suitability of reference sites and ranges of natural variability in field related studies continued to surface, work was also required to determine if these criticisms were valid.

Studies continued in the Moose River Basin at Smooth Rock Falls following fish populations for recovery after process and treatment changes. As well, initial studies were conducted at the pulp mill in Iroquois Falls in 1998. White sucker collected downstream of the mill on the Abitibi River were in very poor condition, as they appeared to be starving. At the time of sampling, the mill in Iroquois Falls had been closed for several months because of a labor dispute. Abitibi River females collected downstream were lighter, had a lower condition factor and smaller gonads, and showed lower size-at-age relative to a number of reference sites (Munkittrick et al., 2000a). Males also had reduced condition factors and reduced gonadal development. Examination of male gonadal development demonstrated that as male fish from the exposed site got longer, their gonad size actually got smaller (Munkittrick et al., 2000a). Apparently the elimination of the nutrient input from the mill effluent reduced the food available for the sucker population resulting in the poor condition of the fish. Collections as part of their Cycle 2 EEM studies conducted during the fall of 1999 attempted to use white sucker as one of their sentinel species, however very few white sucker were captured downstream of the effluent discharge (C. Portt, pers. comm.).

As part of the Moose River studies designed to evaluate the impacts of pulp and paper mill effluents on exposed fish populations, studies were also conducted to evaluate and understand the degree of variability inherent in reference or unexposed populations. This variability was examined among populations from different rivers in the Moose River Basin (spatial comparisons) and within select populations over time (temporal comparisons). Studies conducted in 1994 at five reference sites of similar habitat on different rivers determined that performance characteristics of white sucker were similar among sites (Munkittrick et al., 2000a). For temporal comparisons, white sucker collected upstream of Smooth Rock Falls in 1991 and 1993 to 1998 were compared. Unlike what was demonstrated for the 1994 reference site comparisons, fish performance at one reference site collected during different years, demonstrated significant differences in a number of parameters.

Reproductive studies continued at both the Moose River Basin and at Jackfish Bay attempting to identify in greater detail the mechanisms responsible for the reproductive effects. Previous studies at Jackfish Bay identified a reduced steroid biosynthetic capacity of gonadal tissue in exposed fish as one of the major impacts on reproductive function. Further studies indicated that increases in the level of gonadal apoptosis (programmed cell death) may contribute to these reductions in steroid production at Jackfish Bay (Janz et al., 1997). Janz et al. (2001) followed up this study with comparisons of follicular cell apoptosis and heat shock protein levels in fish collected from the Moose River Basin. In 1996, vitellogenic white sucker collected downstream of the bleached kraft mill in Smooth Rock Falls had reduced ovarian size associated with increases in ovarian cell apoptosis and stress protein (Janz et al., 2001). Fish sampled in 1998 following a number of mill process changes aimed at minimizing the discharge of process chemicals showed no differences in ovary size, apoptosis or heat shock protein levels. Although the specific effluent components that were reduced or eliminated following mill maintenance changes were not known, the changes were associated with significant recovery of these responses compared to 1996. Further research was needed to examine the potential linkages between cellular responses (apoptosis, heat shock protein-70 expression and steroidogenesis) and intergrated responses at higher levels of biological organization (fecundity, ovary size, atresia) (Janz et al., 2001).

Earlier studies by Oakes and Van Der Kraak (2003) had demonstrated that oxidative stress was associated with exposure to pulp and paper mill effluent at locations where reproductive impairment of fish in the receiving environment had been observed. Oakes et al. (2003) then investigated oxidative stress and reproductive impacts in wild white sucker (*Catostomus commersoni*) populations exposed to the effluent in Jackfish Bay, Smooth Rock Falls and Kapuskasing. Since both free radical damage and reproductive impairments were increased with pulp and paper mill effluent exposure, it was possible that free radicals may contribute to the reproductive measures altered with exposure. While the results suggested oxidative stress was not the sole mechanism impairing reproduction, it was likely an important contributing factor (Oakes et al., 2003). Free radicals can also inhibit steroidogenesis by blocking intracellular transport of cholesterol by impairing production of the steroidogenic acute regulatory protein (StAR). As StAR regulates steroid hormone biosynthesis by regulating cholesterol transport, impairments in this protein production could produce the depressed levels of circulating steroids seen in pulp and paper mill effluent exposed fish. Collaborative studies are continuing developing methods to measure StAR in white sucker samples that have been archived over the years from Jackfish Bay (McMaster, pers. comm.).

Detailed studies at Jackfish Bay continued in attempts to identify the chemicals responsible for the reproductive alterations. As effluents are very complex in nature, Hewitt et al. (2000a) developed new methods in attempts to examine only the chemicals that were bioavailable to the fish. Through extractions of liver tissue obtained from both control and previously exposed fish, he was able to identify liver fractions that induced MFO activity as well as those that bound to estrogen and testosterone receptors and to sex steroid binding proteins that carry steroids within the blood (Hewitt et al., 2000a) (See *Characterization of Bioactive Chemicals*).

Following the Northern River Basins Study, which identified evidence of sex steroid depressions in fish and an increased number of sexually immature fish below some of the pulp and paper mills within this basin (Report to the Ministers, 1995), a detailed endocrine evaluation of wild fish in these areas was initiated. This study, funded through Canada's Northern Rivers Ecosystem Initiative and the Toxic Substances Research Initiative, utilized techniques that had been developed over that last number of years of study at Jackfish Bay and the Moose River Basin. This included the measurement of circulating sex steroid levels, gonadal steroid biosynthesis, circulating vitellogenin levels, ovarian apoptosis, hepatic and gonadal oxidative stress, sex steroid binding proteins, gonadal histology (for potential intersex) as well as the stage of gonadal development, hepatic mixed function oxygenase activity, as well as the normal measures of reproductive health including gonadosomatic indices and fecundity estimates. With this large array of endocrine endpoints being analyzed simultaneously, the impact of modern pulp and paper mills in Canada on endocrine-related functions in fish in these receiving environments was determined. Studies were initiated in the fall of 1999 and continued through 2002.

3.6.1 Cycle 2 EEM Collaborative Research Studies

Following Cycle 1 of the EEM program, the Fish Survey Expert Working Group identified a number of key issues that resulted in poor results including an inability to catch sufficient numbers of fish, uncertain exposure, poor reference site selection and potential confounding factors. They also made a number of recommendations for Cycle 2 including a decision tree that included a research option (Fish Survey Expert Working Group, 1997). Collaborative research projects between Environment Canada and industry were initiated to address both mill specific questions following cycle 1 results, as well as some of the larger EEM key science issues. One large collaborative project involved Environment Canada (National Water Research Institute, Burlington Ontario) and multiple mills (James Maclaren Inc. - Thurso and Masson mills and Bowater Pulp and Paper Canada Inc - Gatineau newsprint mill (formerly Avenor Inc)) on the Ottawa River. These studies examined a number of questions including the potential for the use of forage fish in the EEM program (Table 1)(Figure 1)(McMaster & Munkittrick, 2000; McMaster et al., 2000a, 2002a). These fish are often quite small and it was unclear as to whether all of the required EEM fish parameters such as gonad weights and liver weights could be obtained. It is generally assumed that these fish species may also be less mobile and may be useful in separating out other confounding factors or effluents and issues of uncertainty of exposure in fish species. Fish collections were also designed to evaluate reference site variability in the required EEM fish parameters of the species used at these sites. In depth studies examining in mill process lines using short-term laboratory exposures were also conducted at one of these mill locations in collaboration with Noranda/Maclaren (Parrott et al., 2000a) (See *Development and Application of Bioassays*).

These studies demonstrated that fish less than 1 gram total body weight (johnny darter *Etheostoma nigrum*) could be utilized for EEM studies if sampled at the appropriate stage of gonadal development. They appeared to be less mobile and were capable of separating out responses from the various discharges including a downstream sewage discharge (McMaster et al., 2002a). Three reference sites were used for these studies (one for each mill), each with different habitat characteristics. Comparison of the reference fish for each of these mills identified a number of differences in the EEM fish parameters. This indicated that selection of a reference site for forage fish species was critical for EEM type studies and that further study using these forage fish species was needed in order to evaluate their responses. A larger fish species was used as the second sentinel (yellow perch *Perca flavescens*) and a comparison of 5 reference sites was conducted, four from one mill, one from another of the mills on the Ottawa River. Female yellow perch from the first four sites were similar in all aspects evaluated and only a small difference in condition factor was seen in male perch at one of the four reference locations (McMaster et al., 2002a). However, female perch collected at the additional reference site located approximately 40 km upstream of the Thurso mill, demonstrated significantly increased gonadal development relative to the other four Ottawa River reference sites (McMaster et al., 2000a). Upon further evaluation however, this was not surprising as 90% of the water flow at that site consisted of water from the Gatineau River that emptied into the Ottawa River just upstream of this location. Increased gonadal development in these fish could be due to altered food sources or the increased water temperature at this location. Again, these studies indicate that reference site selection is very important for field related studies, however if selected appropriately, definitive conclusions could be made using field studies.

Cycle 2 results were also compared to the Cycle 1 studies conducted at the three mills. Similar to the majority of the mills in Canada, all three of the mills had installed secondary treatment facilities following the Cycle 1 studies in response to the new regulations. One of these mills also conducted a number of in mill process changes including the removal of 80% of the nonylphenol ethoxylates from the process chemicals, increased collection and treatment of foul condensates as well as the initiation of better mill communication and management resulting in reduced black liquor losses (McMaster et al., 2002a). Although direct comparisons could not be made between Cycle 1 and Cycle 2 studies for all of the sentinel species collected, Cycle 2 results indicated improvements in fish health downstream of each of the mills (McMaster & Munkittrick, 2000; McMaster et al., 2000a, 2002a). Both of the two sentinel species used in this research program were also capable of separating out the various confounding effluent discharges at these sites.

As part of the 1996 Cycle I EEM report for the Fraser Inc., Edmundston pulp mill (Edmundston, New Brunswick, Canada), the adult fish survey identified a number of responses in fish downstream of that mill discharge (BAR Environmental, 1996). Yellow perch collected downstream of the pulp mill diffuser had lower growth compared to fish from upstream reference sites. Gonads in male yellow perch were also smaller and females were less fecund. White sucker below the mill had greater growth rates and gonad size, but lower indices of liver size. The Saint John River near Edmundston receives effluent from a pulp mill, a paper mill, three treated sewage discharges, and other untreated sewage releases enter the river over a 10-km reach. In addition, two tributaries carry agricultural runoff into the system. Fraser Papers Inc. (Edmundston pulp mill, Edmundston, New Brunswick, Canada), Nexfor (Noranda Technology Centre, Pointe-Clair, Quebec, Canada), the University of New Brunswick, and Environment Canada (National Water Research Institute, Saskatoon, Saskatchewan and Burlington, Ontario, Canada) participated in a variety of studies as part of Fraser Papers Inc. commitment to EEM studies for cycle 2. The objectives of one study were to develop a database to identify the background fish performance levels of the upper Saint John River basin, to examine whether the differences in fish reported in 1996 were still present in 1999, and to examine the selection of reference sites for these studies (Table 1)(Figure 1)(Galloway et al., 2003). Additional on-site projects included a 90-150 day flow-through fathead minnow (*Pimephales promelas*) bioassay (Parrott et al., 2000b, 2003a,b; See *Development and Application of Bioassays*), and a flow-through microcosm invertebrate exposure (Culp et al., 2003). Additional studies documented the chemicals in pulp mill effluent that could be accumulated by fish (Hewitt et al., 2003) (See *Characterization of Bioactive Chemicals*). Together, results from the fish survey (Galloway et al., 2003) and the microcosm flow-through invertebrate exposure (Culp et al., 2003) suggested that improved performance in wild fish, in terms of energy storage and utilization, was related to increased nutrients. Mesocosm studies also were capable of separating out the effects of the multiple stressors in the system (Culp et al., 2003). The wide range of reproductive alterations in the fathead minnow (*Pimephales promelas*) flow-through bioassay (Parrott et al., 2003a,b) however, suggested that compounds capable of producing these effects were still present in the effluent, but that the receiving environment allowed for protection of wild fish species at this location. Fish tissue extracts from caging studies also demonstrated endocrine potential of the effluent (Hewitt et al., 2003).

3.6.2 4th International Conference on Environmental Impacts of the Pulp and Paper Industry.

The 4th International Conference on environmental impacts of the pulp and paper industry was held June 12-15, 2000 in Helsinki, Finland. In the first three conferences, the main emphasis was on the aquatic environment. During the past decade, the emissions to the receiving waters have continuously decreased despite the increase in production. The 4th conference focused on the integrated approach of impacts originating from effluents, solid wastes and airborne emissions of the pulp and paper industry. In comparison to the 3rd international conference in New Zealand there was a definite shift from biological studies back to chemistry. This was because of the large number of chemistry studies related to process changes that have occurred at mills around the world over the last number of years. Although the number of studies that examined the biological responses to effluent was reduced, they consisted mostly of studies examining chronic toxicity of effluents and larger integrated assessments.

One of the major questions asked of all ecological field studies that have demonstrated potential reproductive alterations in fish exposed to pulp and paper mill effluents is, are the reproductive effects real and what is the ecological relevance of the changes? Munkittrick et al. (2000b) examined the reproductive performance of fish at Canadian mill sites over the period of mill modernization (1989-1999) to address these questions. He used the studies from the Moose River Basin designed to develop methods for cumulative effects assessments for this purpose. The Moose River studies examined fish at 20 different sites, through a series of 50 different fish collections on 6 different tributaries within the basin. It included reference site comparisons looking at longitudinal variability between sites on adjacent tributaries upstream of the major developments, the latitudinal variability within a river as distance downstream increased and examination of seasonal and annual variability within sites.

Although *statistically significant* variability in fish performance existed between sites and among years, one could still evaluate the ecological significance of changes at some sites. There are three common approaches to assessing whether changes are ecologically relevant (reviewed in Munkittrick et al., 2000b):

a) Changes are more than two standard deviations (2 SD) from the reference condition,
b) Changes are outside the range of normal variability (maximum and minimum reference condition),
c) Changes are outside a predefined maximum tolerable difference (i.e., 25% for gonad size).

These values were calculated using the multitude of reference collections for this study and ecologically relevant differences were detected at both undeveloped reference sites as well as downstream of some of the developed sites within the basin (Munkittrick et al., 2000a,b). The key findings were that a) changes in gonad size, liver size and condition factor can be ecologically relevant at some sites, b) process changes have a larger impact on reproductive performance than installation of secondary treatment, c) some reproductive impacts persist at some mills after modernization and waste treatment, d) secondary treatment is very effective at reducing liver sizes and can improve condition of the fish, and e) undeveloped sites with fish showing poor performance can be found in other rivers, but upstream and downstream comparisons are essential to understanding the local relevance of changes (Munkittrick et al., 2000a).

3.7 Evolution of the Research Questions: Cycle 2 EEM Results, What Were the Major Response Patterns and How Widespread Were They?

Industry and the research community now agreed that reproductive effects had been demonstrated downstream of some mills at numerous places around the world and that partial recovery had occurred at a number of locations following process and treatment upgrades. How widespread the responses were however was still at question. Results from Cycle 2 of the Canadian EEM program were received by Environment Canada in April of 2000. This represented the first collections following the installation of secondary treatment at the majority of the mills in Canada and would provide a first glance at the responses of the receiving environments across the country. Cycle 1 data were used as a baseline to gain a better understanding of the variability of the field measurements. This lead to an improvement of Cycle 2 study designs and gave some insight into the typical response patterns of fish and benthos to pulp and paper mill effluents. Cycle 2 proved to be the first EEM cycle with consistently good data, allowing Environment Canada to begin addressing the fundamental objective of the EEM program: *… to determine the potential effects of pulp and paper mill effluents on fish populations and benthic invertebrate communities.* Environment Canada identified a number of questions for data analysis based on priorities set forth by the department, the pulp and paper industry and other partners.

A national assessment of EEM data was conducted with the objective of presenting the current state of aquatic ecosystems around Canadian pulp and paper mills and to present any discernible trends in the receiving environment over the past 10 years. In doing so, the report presents in depth information on the state of the fish populations and the benthic invertebrate communities existing in the receiving environments across Canada. More specifically, the report addresses a number of priority areas identifed by government

EEM scientists through consultation with industry such as how the magnitude of effects and sublethal endpoints are influenced by mill process, effluent treatment, and habitat/community type of the receiver.

This assessment examined the national response patterns demonstrated in both the benthic invertebrate communities and the fish surveys. The magnitude and direction of effluent-associated effects on benthic invertebrate communities varied significantly among different habitat types. Abundance was significantly increased in both erosional and depositional riverine habitats. Taxon richness was not significantly changed when considering riverine habitats as a whole (Lowell et al., 2003). Further subdividing the riverine data set revealed that the distribution of individual mill responses for taxon richness was weighted toward mills showing significant increases and those showing no significant change. Thus, the average response pattern observed in riverine exposure areas was one of mild to moderate eutrophication. In contrast, both abundance and taxon richness decreased in marine/estuarine habitats, a response that was indicative of toxicity or a smothering response pattern (smothering effects are the result of the deposition of pulp fiber mats). The distribution for lacustrine habitats was intermediate between that for riverine and marine/estuarine habitats (Lowell et al., 2003). Mild to moderate eutrophication effects have been well documented in smaller-scale studies for mills discharging into riverine habitats (Hall et al., 1991; Culp et al., 2000; Lowell et al., 2000), and riverine habitats comprise the majority (two-thirds) of the mills included in the invertebrate analyses for Cycle 2. One of the advantages to the large-scale meta-analytical approach reported was that it had identified differences between freshwater and marine responses that were not previously documented. This led to a variety of new testable hypotheses on habitat-related effects: historical deposition of fiber mats (are fiber mats flushed less regularly in marine than in freshwater habitats), species sensitivity (are there differences among habitat types in taxa sensitivities to effluent exposure), multiple co-occurring effects (are freshwater inhibitory effects more often masked by eutrophication), and study design (is there a difference in positioning of sampling stations, relative to outfalls, in freshwater versus marine habitats) (Lowell et al., 2003).

The fish survey was designed to measure key endpoints related to age, energy use (growth rate, reproductive investment) and energy storage (condition factor, liver size) (Lowell et al., 2003). An eutrophication response would be expected to result in increased energy use and storage, and this was indicated by some components of the national average response seen in fish (increases in weight at age, condition, liver weight). In contrast, an average decrease in gonad size was also found that, in combination with the increases in the other fish endpoints, was similar to patterns previously observed at some pulp and paper mills prior to the initiation of the EEM program (Munkittrick et al., 1994a). This pattern of decreased gonad size, combined with increased energy use and storage, had been interpreted as a form of metabolic disruption (Munkittrick et al., 2002a) or endocrine disruption (Van Der Kraak et al., 1992), associated with difficulties in producing sufficient sex steroid hormones (McMaster et al., 1996b). Male and female fish showed similar responses for all endpoints, with significantly greater decreases in gonad weight being observed for female fish ($P < 0.05$) (Lowell et al., 2003).

Improvements in effluent treatment and pulping processes had resulted in some recovery from the reductions in gonad size associated with pulp mill effluents in the early 1990s, and it was clear that not all pulp mills were associated with ecologically significant impacts on fish (Munkittrick et al., 2002a). The finding, however, of an average national pattern consistent with early studies on a limited number of pulp and paper mills suggested that there were still potential concerns regarding pulp and paper mill effects on the reproduction of fish via alterations in levels of sex steroid hormones. At the same time, the measured increases in energy use and storage suggested that there was sufficient food for the fish, consistent with the eutrophication responses evident in the invertebrate studies (Lowell et al., 2003).

A special EEM theme issue of the *Water Quality Research Journal of Canada* outlined some of the key EEM research issues in a peer-reviewed forum in order to increase the circulation and availability of the information developed over the last number of years (Munkittrick et al., 2002b). In addition to an overview of the pulp and paper program, the issue included a variety of papers describing results from the first two EEM cycles including papers describing some of the concerns related to data interpretation and sampling frequency, the development of alternative approaches as well as non-lethal sampling methodologies (Munkittrick et al., 2002b). The National EEM Science Committee developed a variety of research needs based on the experiences of the scientists after the first two cycles of EEM monitoring. In freshwater, the highest research priorities related to fish monitoring were related to developing non-lethal sampling methodologies and developing additional information on home ranges, mobility and exposure of small-bodied forage fish species. As noted after cycle 1, there was also a need to develop additional information on the basic life history of these species, and on how to estimate reproductive performance of multiple spawners using a single sampling event, and the potential sampling designs for multiple spawners using sequential sampling (Munkittrick et al., 2002a).

For fish studies in the marine environment, significant progress was made between Cycles 1 and 2. On the Atlantic coast, it was determined that the small bodied, littoral mummichog (*Fundulus heterclitus*) could be easily and quickly sampled and provided all of the information required by the Fish Survey. It was present at six of the mills, and at the other mills other small-bodied species such as rock gunnels and cunners were present (Courtenay et al., 2002). For the Pacific coast, small-bodied littoral species would likely play little, if any, role in cycle 3. Unlike the Atlantic coast where many mills discharge effluent into estuaries and shallow water near shore, most B.C. mills discharge effluent at depth and have trapped plumes. For some of these sites, surveys of wild bivalves or snails may be suitable, whereas at other sites caged bivalves may be the best option. Areas requiring continued research in the marine environment included better methods for the collection of data on age, sex and reproduction in bivalves and in other potentially useful sentinels such as the gastropod-molluscs sampled in Quebec during cycle 2 (Courtenay et al., 2002).

Kovacs et al. (2002) conducted studies on the St. François River to assess the biological condition of fish in the vicinity of three pulp and paper mills using three approaches different than what is used for the EEM studies (Table 1). It was there intent to determine whether the use of a characterization of the fish community structure using the Index of Biotic Integrity (IBI) predicted improvements in receiving water quality better than a fish health assessment index (HAI) or the measurement of two biochemical parameters, hepatic mixed function oxygenase activity and plasma steroid levels. Using the IBI approach, they compared collections conducted in 1991 by the Quebec Ministry of the Environment (Richard, 1996), with collections they conducted in 1998. During this time, two of the mills installed secondary effluent treatment systems, while the third mill already had secondary treatment of their effluent. The 1991 study documented reductions in the IBI downstream of the two mills that had only primary treatment of their effluents, but demonstrated increases in IBI downstream of the mill with secondary treatment (Richards, 1996). The 1998 collections demonstrated improvements in the fish communities downstream of the two mills that installed secondary treatment (Kovacs et al., 2002). The IBI downstream of the third mill that had secondary treatment during both collections also showed improvement in 1998, which corresponded to altering the processes somewhat between collections, with corresponding reductions in BOD and TSS (Kovacs et a., 2002). However, the IBI index was still higher downstream of the discharge relative to upstream.

In comparison, the collections for the other two methods (HAI and biochemical parameters) were conducted in September 1995, one month following the installation of secondary treatment at the two upstream mills. For the 1998 collections, the HAI approach demonstrated conflicting results with the two species used at two of the mills. Where tessellated darters (*Ethoeostoma olmstedi*) were used in 1998, HAI values were actually reduced downstream of the mill discharge, but in comparison, white sucker demonstrated increased HAI values (Kovacs et al., 2002). The biochemical parameters failed to demonstrate any mill related responses in terms of decreased steroid levels or increased MFO activity in 1998 suggesting that either the compounds responsible for these effects were not present or that the receiving environment protected fish from these chemicals. All mills on this system had secondary treatment during all collections for the biochemical comparisons, and other authors have documented recovery in these endpoints following the installation of secondary treatment at other locations (Sandstrom, 1994, 1996; Munkittrick et al., 1997a). Unfortunately the authors failed to compare the three approaches to the EEM sentinel species approach that was also used at these three sites for their cycle 1 and 2 collections. They conclude however that the IBI community approach was capable of demonstrating improvements following the installation of secondary treatment at two sites and process changes at the third location.

Studies initiated in attempts to determine whether effluents from modern mills also caused reproductive alterations in wild fish within the Northern River Basins were completed. Fish health was examined and compared to a number of reproductive endpoints developed to assess endocrine function. As these Northern Rivers were nutrient limited, fish responded to effluent addition (increased nutrients) by showing signs of increased growth and development relative to the upstream reference fish (McMaster et al., 2000b, 2002b). Some signs of altered reproductive function were observed downstream of the mill sites, however additional responses were observed downstream of the municipal sewage treatment plants from the neighbouring communities. For example, longnose sucker collected downstream of the municipal sewage discharge from the city of Grande Prairie, demonstrated alterations in circulating steroid hormone levels and vitellogenin levels relative to the upstream reference fish (McMaster et al., 2002b). The reproductive responses at these three mills were minimal compared to earlier studies examining reproductive function in fish exposed to pulp and paper mill effluents in Jackfish Bay on Lake Superior and in Kapuskasing and Smooth Rock Falls in the Moose River Basin. In fact, in terms of EEM monitored endpoints, fish either showed no responses or a response to nutrient enrichment with increased growth, condition, liver size and gonadal development (McMaster et al., 2002b). Studies were also conducted at these locations examining effluents from these sites for endocrine active compounds (See *Characterization of Bioactive Chemicals*). Effluents from each of the three mills examined in the wild fish studies contained compounds that bound to both estrogen and androgen receptors as well as to sex steroid binding proteins. Whether the nutrient enrichment response in the fish masked the potential for reproductive effects however was not known.

3.8 Conclusions

In response to the results from the Swedish studies in the early 1980s, a number of Canadian studies were initiated to determine whether similar responses were seen downstream of pulp mills in Canada. This was confirmed at a number of locations, however not all studies demonstrated effects in the receiving environment. Following the confirmation of effects, a new regulatory package for the pulp and paper industry was introduced which set new levels for BOD, TSS and acute lethality as well as limits for dioxins and furans. In order to meet the new regulatory limits, a number of process and treatment changes were required by industry. Research continued and determined that reproductive impacts in fish were not limited to mills that used chlorine in the bleaching process and were present in some mills with secondary treatment. Detailed mechanistic studies identified alterations in the gonadal steroid biosynthetic capacity as the major site of reproductive action. Studies continued at a number of locations and some signs of recovery were demonstrated following both process and treatment changes, although the specific role of each in the recovery was unknown. As part of the new regulatory framework, an Environmental Effects Monitoring program was also initiated which required mills to conduct receiving water fish and benthic studies. Following the second cycle of the program, a national assessment was conducted identifying national response patterns in both benthic and fish endpoints. The finding of an average national pattern consistent with the early studies on a limited number of pulp and paper mills suggested that there were still potential concerns regarding pulp and paper mill effects on the reproduction of fish via alterations in levels of sex steroid hormones. At the same time, the measured increases in energy use and storage suggested that there was sufficient food for the fish, consistent with the eutrophication responses evident in the invertebrate studies.

4. Development and Application of Bioassays

4.1 Summary

The development and application of bioassays for pulp mill effluents (PMEs) have evolved over the past decade with the evolution of the research questions. Figure 3 shows the evolution of the research questions, and the resulting evolution of laboratory bioassays. As the issues of acute lethal toxicity of final PMEs were resolved, the focus moved from short-term acute assays to sublethal tests.

Laboratory tests have been used to answer many of the regulatory and research questions that have arisen related to the effects of pulp and paper mill effluents on aquatic biota. Acute, short-term, laboratory tests have been used to assess final effluents and to track changes in effluent quality over time. Short exposures of fish and invertebrates form part of Federal and Provincial effluent discharge regulations for pulp and paper mills and other industries. Short-term laboratory tests have clearly shown the improvement in final effluent quality following secondary treatment of effluent.

In an effort to predict and investigate impacts on wild fish, laboratory bioassays have been developed to examine MFO induction and steroid depression. Exposures of fish in the laboratory have been used to identify pulp and paper mill-related chemicals that induce MFO and suppress steroids. Short-term laboratory exposures of fish have been used to assess responses of fish to pulp and paper mill effluents, to specific components of pulp and paper mill processes (wood extractives, black liquor) and to discriminate between historical and present day discharges.

These short-term bioassays have enabled specific responses, such as MFO induction or steroid depression, to be linked to mill process streams or waste products. Assessment of MFO induction and steroid reduction caused by pulp and paper mill effluent has enabled comparison of types of mill processes, wood furnish, and effluent treatment. It appears that MFO induction and steroid depression are found in fish exposed to effluent from a variety of mills types, with and without chlorine bleaching, in hardwood and softwood pulping facilities, and before and after effluent treatment.

New genomic techniques have contributed to the ability of laboratory bioassays to detect changes associated with exposure to pulp and paper mill effluents. The development of chromosomal markers to determine the genetic sex of some fish species has led to some novel findings: after 1-month exposure to high concentrations of bleached kraft mill effluent, some male fish had ovaries. Advanced techniques have shown that pulp and paper mill effluent decreases sex steroids in exposed fish, but the gene expression pattern is different than fish exposed to estradiol. These genetic technologies may lead to the development of shorter bioassays that are linked to reproductive effects.

Figure 3. Timeline and evolution of research questions for aquatic bioassays to assess the impacts of pulp and paper effluents in Canada.

1990	Effluents from bleached kraft pulp and paper mills declared toxic under the Canadian Environmental Protection Act
1991	Do short- term lab assays predict the field effects of importance?
	Can we develop a short- term assay for MFO induction?
1992	Can we replicate impacts using laboratory life-cycle tests?
	What are the most appropriate endpoints for monitoring reproduction of fish exposed to pulp mill effluents?
1993	Can we develop a short- term assay for predicting steroid hormone changes?
	How do effluents from different mills affect MFO activity?
1994	What is the potential of effluents from different pulping processes to affect fish reproduction?
	What effect does process change have on reproduction of fish in long term laboratory exposures?
1995	Can we develop a short-term assay for steroid responses that can be used to direct chemical manipulations of effluents?
	What are the laboratory responses to wood components?
	How are pulp mill effluents related to the emerging endocrine disruptor controversy?
1996	Which effluent streams or mill processes are associated with steroid depressions?
1997	Is there laboratory evidence for a reduction in the toxicity of effluents?
1998	What is the potential of effluents before and after treatment and from different process types to affect sex steroid signaling?
	Can we develop an on-site bioassay protocol for improving our ability to investigate waste process streams?
	Can we shorten a life cycle test that would detect effluent responses?
1999	Do fish exhibit evidence of exposure to androgenic compounds in effluents?
	What is the relative sensitivity between on-site life cycle exposures and wild fish?
2000	Do short-term assays of sex steroids predict reproductive changes in full life cycle exposures?
2001	How does the response of different mill types compare?
2002	What is the relative sensitivity of laboratory species and native species?
	What is the relevance of the new OECD EDC-testing bioassays for pulp mill monitoring?

Bioassays have been used extensively over the last decade to examine the effects of pulp mill effluents on fish. Longer-term, lifecycle laboratory tests and mesocosm exposures have been used to assess the effects of chronic exposure: growth enhancement of invertebrates and fish, liver enlargement, and decreases in gonad size and fecundity in several fish species. Short-term laboratory tests have assessed individual mill waste streams and have provided information to isolate and treat selected pulp and paper mill wastewaters. Short-term tests have also been used to drive causal investigations in the isolation and identifcation of bioactive compounds.

The most sensitive endpoint in these lifecycle and long-term fish exposures is decreased reproduction. This endpoint is biologically meaningful, but determining thresholds for effects requires lengthy and expensive tests. As the laboratory tests move forward into the next decade, attention will focus on the reproductive endpoints, and the possibility of shortening the fish bioassays while still maintaining sensitivity and biological relevance.

4.2 History

4.2.1 Acute bioassays in EEM

Controlled laboratory exposures of fish and invertebrates to pulp mill effluent (PMEs) have been part of the EEM program for the past decade (Figure 3). The purpose of the short-term assays was to assess potential acute effects of exposure to final effluent, and to monitor changes in effluent quality as the mills implemented treatment and process changes over time. Acute bioassays were conducted twice a year.

The fish short-term assays measure survival and growth in native species exposed to effluent for a week. Fish species used include fathead minnows (*Pimphales promelas*) or rainbow trout (*Oncorhynchus mykiss*) at pulp and paper mills discharging into freshwater environments, and inland silverside (*Menidia beryllina*) or topsmelt (*Atherinops affinis*) at pulp and paper mills discharging into marine environments. Invertebrate assays examined survival, growth and reproduction/fertilization success in invertebrates exposed to PME for up to 3 weeks. Test organisms used in the invertebrate assays were *Ceriodaphnia dubia* at pulp and paper mills discharging into freshwater environments, and gametes of sea urchin (*Strongylocentrotus purpuratus*) or sand dollars (*Dendraster excentricus*) at pulp and paper mills discharging into marine environments (Environment Canada, 1992a, b, c, 1998b).

Results of these acute studies showed that implementation of secondary treatment greatly improved the quality of Canadian PME: There were fewer impacts on fish and invertebrate survival and growth as mills installed secondary treatment (Environment Canada, 1997a, b; Scroggins & Miller, 2000; Lowell et al., 2003).

Short-term laboratory tests were assessed for their ability to predict the impact of final effluents on invertebrates and fish in receiving environments. Borgmann et al. (2001) showed that in data collected during the EEM program, invertebrate tests agreed with results from benthic field surveys in three case studies involving seven Ontario pulp mills. As well, fish acute test results agreed well with impacts on wild fish at most of the 16 Ontario mills (Borgmann et al., 2001).

4.2.2 *Short-term exposures assessing MFO and steroids*

Although acute lethality and growth bioassays could assess short-term effects of pulp and paper mill effluents, it was obvious that better tests were needed, as wild fish health was affected in receiving environments even though the mill's final effluent routinely passed all acute toxicity tests (Figure 3). In an early review of to PME toxicity test data, Kovacs and Megraw (1996) found no consistent patterns in laboratory toxicity test results conducted at several pulp mills utilizing different processes and effluent treatment. Robinson et al. (1994) saw no effects on survival or growth of fathead minnow larvae exposed for 7 d to 14 different pulp and paper mill receiving waters, even though wild fish showed changes in growth and reproductive indicators at several mill sites (Munkittrick et al., 1994a). It should be emphasized that Robinson et al. (1994) tested receiving water, not final mill effluent. Performance of laboratory tests is maximized when final effluents are tested in a series of dilutions, so that a dose-response may be assessed and threshold effluent concentrations may be estimated.

The development of short-term sublethal tests to assess the impacts of pulp and paper mill effluents was based on the observations in wild fish that were common to most pulp and paper mill sites: MFO induction and steroid depressions. Some of the earliest controlled exposures of fish to pulp and paper mill effluent involved caging wild fish in pulp mill effluent streams. These caging studies were useful in determining the short-term responses to pulp mill effluent (MFO induction, steroid depression), and were the first step in progressing from field observations to short-term predictive laboratory tests.

Measurement of MFO enzymes was one of the first biomarkers to be used in laboratory bioassays of fish exposed to pulp mill effluents. To assess the MFO-inducing potential of PMEs, ethoxyresorufin-O-deethylase (EROD) activity in fish liver homogenates was measured after exposures as short as 4 days (Hodson et al., 1996; Parrott et al., 1999).

White sucker captured from a reference site and exposed to bleached kraft mill effluent (BKME) in cages or 80-L containers had dramatic MFO induction (Munkittrick et al., 1999; Parrott et al., 1999). Immature white sucker and juvenile rainbow trout exposed to BKME had dramatic MFO induction (Munkittrick et al., 1999). However, goldfish caged in 50 % BKME showed no MFO response (McMaster et al., 1996a). This may be due to the recalcitrant nature of goldfish to MFO inducers. Kidd et al. (1993) had to intraperitoneally dose goldfish with 50 µg/kg PCB126 to see even moderate MFO induction.

In-situ caging experiments demonstrated some characteristics of the MFO induction which have provided clues to the properties of the inducing substances (See *Characterization of Bioactive Chemicals*). Studies in 1989 showed that white sucker captured from the effluent plume and held in clean water for 4 d had a marked decrease in liver MFO activity (McMaster, 1991). Exposures of juvenile white sucker to BKME showed EROD induction peaked in 8 d, and remained elevated until 14 d, whether or not the fish were removed to depurate in clean water. EROD activities returned to pre-exposure levels after 24 days (Munkittrick et al., 1994b, 1999).

Laboratory exposures of fish confirmed that EROD activity was induced by effluent from pulp and paper mills of various production types. In a survey using 31 samples of secondary-treated PMEs from eight different mills, Martel et al. (1994) found that rainbow trout MFO was most consistently induced after exposure to kraft mill effluent (both bleached and unbleached). In a broader examination of MFO induction by various PMEs, rainbow trout exposed for 4 d to 10

% final effluent showed significant MFO induction in 29 of 46 effluent samples tested (Martel et al., 1996). Induction was seen in effluent from several types of pulp and paper mills: unbleached and bleached kraft mills as well as thermomechanical and chemi-thermomechanical mills (Martel et al., 1996). MFO induction was seen in trout exposed to primary treated effluent, as well as secondary treated effluent. Ten of the 29 effluent samples caused less than 2-fold induction over control fish MFO. Nineteen of the 29 effluent samples caused higher induction (up to 15 fold) (Martel et al., 1996). Laboratory exposures of juvenile rainbow trout showed induction by final effluent from four bleached kraft mills and one unbleached kraft mill (Williams et al., 1996). Thresholds for significant MFO induction ranged from 0.3 to 9 % final effluent (Williams et al., 1996). Wood condensates in black liquors also induced MFO in laboratory exposures of rainbow trout (Martel et al., 1994; Hodson et al. 1996; Coakley et al., 2001). Four-day exposures to spent bleaching liquors from two Ontario BKMEs (both using ClO_2 bleaching) caused MFO induction (Coakley et al., 2001). Filtrates from softwood bleaching were about 3-fold more potent than filtrates from hardwood bleaching, and filtrates from the last stage of bleaching were more potent than filtrates from the first stage of bleaching (Coakley et al., 2001).

Because the MFO induction in fish is a very rapid response, studies have examined which mill process streams cause induction, as well as which effluent treatments successfully reduce inducing potency of final effluent. Treatment for the removal of hard-to-degrade organics and an activated sludge treatment process consistently removed more than 85 % of effluent-induced MFO activity from a BKME with 60 % ClO_2 bleaching (Schnell et al., 2000). Characterization of various in-plant process waters sampled at the mill indicated that the softwood-line bleach plant was a major contributor (>70 %) to the MFO induction potential of untreated and biologically-treated BKME (Schnell et al., 2000).

Retene (7-isopropyl-1-methylphenanthrene) in pulp mill effluent and sediments has been investigated as one of the potential causes of MFO induction in fish downstream of pulp mill effluents. This substituted phenanthrene is a derivative of resin acids, and can be produced in anaerobic sediments by bacterial metabolism of resin acids (Wakeham et al., 1980). However, resin acids were not able to induce MFO in fish (Ferguson et al., 1992). Retene can be found at concentrations over 3,000 μg/g dry wt in sediments of rivers receiving PME (Oikari et al., 2002). Increased ethoxyresorufin-O-deethylase (EROD) activity was observed in juvenile trout exposed for 4 d to waterborne retene at 100 μg/L (Parrott et al., 1994; Fragoso et al., 1998, 1999). Induction peaked at 4 days, and moving exposed fish to clean water showed elimination of retene as indicated by EROD activities returning to pre-exposure levels (Fragoso et al., 1998, 1999).

Although much research has focused on the identity of the MFO inducer in PMEs, the conclusions are still elusive. Compounds in mill effluents such as retene, chlorinated and unchlorinated diarylethanes (stilbenes; Burnison et al., 1996, 1999), and compounds from wood extracts such as juvabione and dehydrojuvabione (Martel et al., 1997) can all induce EROD, but there is no definitive list of MFO inducers at various mills. It appears that the MFO inducing compounds are a diverse group, and vary with mill process, wood type, and effluent treatment (See *Characterization of Bioactive Chemicals*)

With the search for the MFO inducer came considerable debate over the biological relevance of MFO induction (Martel et al., 1994). MFO induction was associated with but not mechanistically-linked to reproductive effects in wild fish (Hodson, 1996). Evidence mounted that the compounds causing MFO induction and the compounds causing steroid depression in PME may be different groups of chemicals (Munkittrick et al., 1994a). The focus of the laboratory bioassays then shifted to steroid depression and reproductive effects.

Short-term fish exposures were used to assess the steroid-reducing capacity of PMEs. Field-captured fish could be caged in PME and assessed for changes in circulating steroids. Laboratory fish transported to the field and exposed *in situ* were used to assess the steroid-reducing potential of PMEs. Laboratory exposures of small fish species were also used to assess PMEs ability to depress steroids. Laboratory exposures for steroid assessments were usually static-renewal assays, lasting from a week to a month. The steroids measured included testosterone, 11-ketotestosterone and estradiol.

A variety of pulp and paper mill effluents have been shown to decrease fish reproductive indicators (such as GSI, fecundity) and alter circulating steroid hormones (See *Field Studies and Mechanistic Research*). Alterations in reproduction and circulating sex steroid hormones also have been seen in fish exposed to pulp mill effluent in laboratory or mesocosm studies. In general, fish exposed to a variety of pulp mill effluents under controlled conditions in the laboratory have depressed sex hormone levels.

Caging of laboratory test species in BKME streams were the first assays that were able to mimic the reproductive changes and steroid depression seen in wild fish exposed to BKME (McMaster et al., 1996a). Effects on goldfish were most dramatic when exposures were conducted on site, with fish caged in effluent streams. A decrease in potency was noted in laboratory exposures of goldfish to effluent under static, daily renewal conditions. However, McMaster et al. (1996a) propose that this could be due to fish loading density in laboratory exposures, as well as to biological factors (testing of goldfish during reproductive senescence). As well, mill process changes and upgrades make it difficult to assess test sensitivity over time, as effluent quality is continually changing.

Development of techniques for measurement of steroid production by excised gonads of fish (McMaster et al., 1995b) allowed the production of steroids to be assessed in testes and ovaries, under either basal or stimulated conditions (with added human chorionic gonadotropin (hCG), to stimulate the gonad to produce hormones). Techniques for measurement of testicular and ovarian *in vitro* steroid production paved the way for investigations into the effects of PMEs on the reproductive functions of smaller-bodied fish species (too small to sample blood for sex steroid measurement). Fathead minnows and marine mummichogs exposed to PMEs showed decreases in production of sex steroids by excised testes and ovaries (Robinson, 1994; Dubé & MacLatchy, 2000a). In general, measurements of steroids produced by excised gonads correlate well with levels of circulating steroids.

Controlled exposures using laboratory fish have been used to address whether current PME discharges have potential to cause steroid disruptions, or whether historical input may be associated with the effects. Early studies at Jackfish Bay demonstrated depressions of sex steroid hormones in white sucker (Munkittrick et al., 1994a; McMaster et al., 1996b). Goldfish that were transported to this site and exposed to BKME also showed changes in sex steroids after 4-8 d exposures (McMaster et al., 1996a), indicating current discharges were sufficient to cause the reproductive effects. These findings were confirmed at a bleached sulphite mill (BSM) where goldfish exposed for 21 d on-site in 1997 to final effluent also had reduced circulating sex steroids (Parrott et al., 1999, 2000a). At a bleached kraft mill located in Saint John NB, the receiver is a tidal environment with inputs from other industries and it is not possible to conduct wild fish assessments. In this case, mesocosm studies using mummichog were necessary to evaluate the potential of this effluent to affect fish reproduction. Mummichog exposed for a month to 1 % BKME had depressed circulating sex steroid concentrations (Dubé & MacLatchy, 2000a).

The development of short-term laboratory exposures of fish that could detect the steroid-reducing potential of PMEs allowed researchers to search for bioactive compounds in effluents. The search for the compounds responsible for reproductive impairment in fish exposed to PMEs has examined several plant-based compounds, such as *β*-sitosterol and genistein. *β*-sitosterol was detected in a survey of 22 US pulp and paper mill effluents at concentrations ranging from 71 to 535 µg/L (Cook et al., 1996). Goldfish (*Carassius auratus*) exposed to *β*-sitosterol (intraperitoneally injected or waterborne) had decreased plasma steroid concentrations (MacLatchy & Van Der Kraak, 1995; MacLatchy et al., 1995, 1997). *β*-sitosterol (≥ 75 µg/L) reduced the concentrations of plasma testosterone, pregnenolone and cholesterol in immature rainbow trout after 21 d laboratory exposures (Tremblay & Van Der Kraak, 1998). *β*-sitosterol also binds weakly to trout hepatic estrogen receptor (ER) and weakly induces vitellogenin in trout hepatocytes and in fish exposed for 21 d to concentrations of 25-100 µg/L (Tremblay & Van Der Kraak, 1998). However, Tremblay and Van Der Kraak (1998) propose that the steroid reducing and ER-agonist activities represent two different modes of action of *β*-sitosterol, as it acts differently than traditional ER-agonists such as estradiol or nonylphenol. MacLatchy et al. (2002) have shown that brook trout (*Salvelinus fontinalis*) and goldfish implanted with *β*-sitosterol had a decrease in the available gonadal mitochondrial cholesterol pool for the P450 enzyme responsible for side chain cleavage (P450scc), but had no change in the activity of this enzyme (that converts cholesterol to pregnenolone). The proposed mechanism of action for *β*-sitosterol is the impairment of cholesterol transfer across mitochondrial membranes, thereby decreasing pregnenolone and sex steroid levels (MacLatchy et al., 2002).

Another plant-based compound, genistein, was found to alter gonadal development in fish. Genistein is a plant flavanoid, found in the heartwood of many tree species. Concentrations of genistein in final BKME from Ontario were 10 µg/L (Kiparissis et al., 2000). Newly-hatched Japanese medaka (*Oryzias latipes*) exposed to genistein for 3 months had altered secondary sex characteristics and abnormal gonadal development. Histological examination of the gonads showed that male medaka testes contained eggs (a condition called "testis-ova"), and that male fish also showed a feminization of secondary sex characteristics at concentrations of 1,000 µg/L genistein. Female medaka showed masculinization (male-like dorsal and anal fins) and histological examination of the ovaries showed oocyte atresia (Kiparissis et al., 2000).

Recently, short exposures of laboratory fish have shed light on the genes altered by PME exposure that may be related to decreased hormones. Advances in genetic techniques have determined that the gene expression response pattern of fish to a paper mill effluent was different from the effects of estradiol. Largemouth bass exposed for 7 d to 2 months to 10-80 % bleached paper mill effluent had decreased circulating steroids and vitellogenin. Male fish held for 7 d in the effluent showed a different gene expression pattern than male fish exposed to estradiol (Denslow et al., 2000). The authors suggest that the mechanism for the steroid reductions is not through interference with the estrogenic cascade, but through some other pathway (Denslow et al., 2000). As molecular and genetic techniques advance, laboratory bioassays will evolve to assess the reproductive and steroid-reducing capacity of PMEs with efficient and biologically relevant endpoints.

Short-term exposure bioassays have also been used to assess the potential mutagenicity of compounds in PMEs. Easton et al. (1997) exposed Chinook salmon to PME, and found increases in size variability of red blood cell (RBC) nuclei. There was considerable debate over the findings, and the potential link to the health of BC Chinook salmon populations. Exposure to the PME caused fragmentation of the RBC nuclei but not heritable mutagenic and carcinogenic changes. Laboratory studies assessing the ability of PME to cause genetic mutations have shown that solvent fraction of some PMEs can induce back-mutation of *Salmonella* bacteria in the Ames assays (Rao et al., 1994, 1995a, b), and can induce micronuclei formation in rainbow trout (Rao et al., 1995b).

In general, field studies have rarely found increases in neoplasms in wild fish exposed to PMEs. However, one field study has shown increases in DNA adducts in fish exposed to BKME. In the Fraser River, BC, a study of wild Chinook salmon exposed to PME detected increased DNA adducts in liver microsomes, although there were no increases in liver lesions as detected by histology (Wilson et al., 2000).

Laboratory exposures of fish to PME have been able to duplicate the DNA adducts seen in wild fish. Wilson et al., (2001) exposed juvenile Chinook salmon for 28 d to up to 2-16 % final effluent from an elemental chlorine-free (ECF) BKME mill. Hepatic microsomal DNA adducts were elevated in fish exposed to 8 and 16 % effluent, and EROD was induced in fish exposed to effluent concentrations of 2 % and higher. The magnitude of DNA adducts correlated well with the EROD activity, and may suggest a mechanistic link: An increase in biological oxidation (increased MFO) within liver cells may generate reactive metabolites. These compounds may then bind to proteins, lipids and other components of cells (DNA and RNA), and may alter their function. Such studies provide insight into the potential biological significance of MFO induction.

Laboratory studies of mussels exposed to PME have shown similar reproductive changes to fish: suppression of sex steroids and decreases in fecundity. Freshwater mussels (*Elliptio buckleyi*) exposed for 56 d to 40 and 80 % paper mill effluent (from the Georgia Pacific Mill in Palatka, Florida) had decreases in mantle concentrations of estradiol in females and testosterone in males (Gross et al., 2000). None of the female mussels from the 40 and 80 % PME treatment were gravid, compared to 50 % gravid females in control mussels, and 42-46 % gravid females in the 10 and 20 % PME treatments, respectively (Gross et al., 2000). Gross et al. (2000) suggested that mussels may be a sensitive organism to study the reproductive potency of PMEs. Similar decreases in fecundity were seen in fish exposed to this effluent as largemouth bass (*Micropterus salmoides*) showed a decrease in the number of eggs spawned per female at concentrations of 10 % and above (Sepulveda et al., 2000).

Martel et al. (2003) have investigated the use of caged freshwater mussels (*Elliptio complanata*) at three pulp mills. Growth of mussels caged for 60 d at 10 stations along a river in Quebec was found to be a sensitive endpoint. The unbleached kraft mill showed increased mussel growth, the thermomechanical pulp mill showed no difference in mussel growth, and the bleached kraft mill showed decreased growth. The decrease in mussel growth was reflective of the decreased benthic density, richness and diversity downstream of this mill (Martel et al., 2003).

4.3 Mesocosms

The use of mesocosms to assess the effects of PME on aquatic biota has permitted studies of algae, invertebrates and fish, exposed under natural temperature and lighting conditions (Figure 3). Mesocosm studies have shown that PME increases the periphyton biomass and shifts the diatom community structure (Glozier et al., 2002). These effects are usually related to increased carbon and nutrient loads from the effluent, which may increase algal and bacterial growth (Culp et al., 1996). The increased algal and bacterial biomass results in growth-enhancing effects on invertebrates. Increased benthos abundance downstream of these PMEs was seen in many environmental effects monitoring surveys (Ellis & Lowell, 2001; Grapentine et al., 2001; Lowell et al., 2001, 2003).

Mesocosm studies have exposed mummichog for 28 d to control water or 3 % effluent from the bleached kraft mill located in Saint John NB (Dubé et al., 1997). The mesocosm pilot study, exposing juvenile fish and adult males and females, proved successful at this site (Dubé et al., 1997). Mesocosm studies have been used to track the steroid-reducing effects in bleached kraft pulp mill waste streams (Dubé & MacLatchy, 2000a, 2001b). Dubé and MacLatchy (2001a) have shown that installation of reverse osmosis (RO) treatment of evaporator condensates can reduce the potency of effluent to reduce plasma sex steroids and *in vitro* ovarian and testicular steroid production. Adult mummichogs, captured from a nearby reference site, were exposed to final BKME or to RO-feed and RO-permeate for 27 to 57 days. Exposure to 1 % effluent reduced steroids in 1997, but after installation of RO by the mill in 1997-1998, the effluent was not as potent at causing steroid depression (Dubé & MacLatchy, 2000a, b, 2001a) (See *Characterization of Bioactive Chemicals*).

4.4 Lifecycle Studies

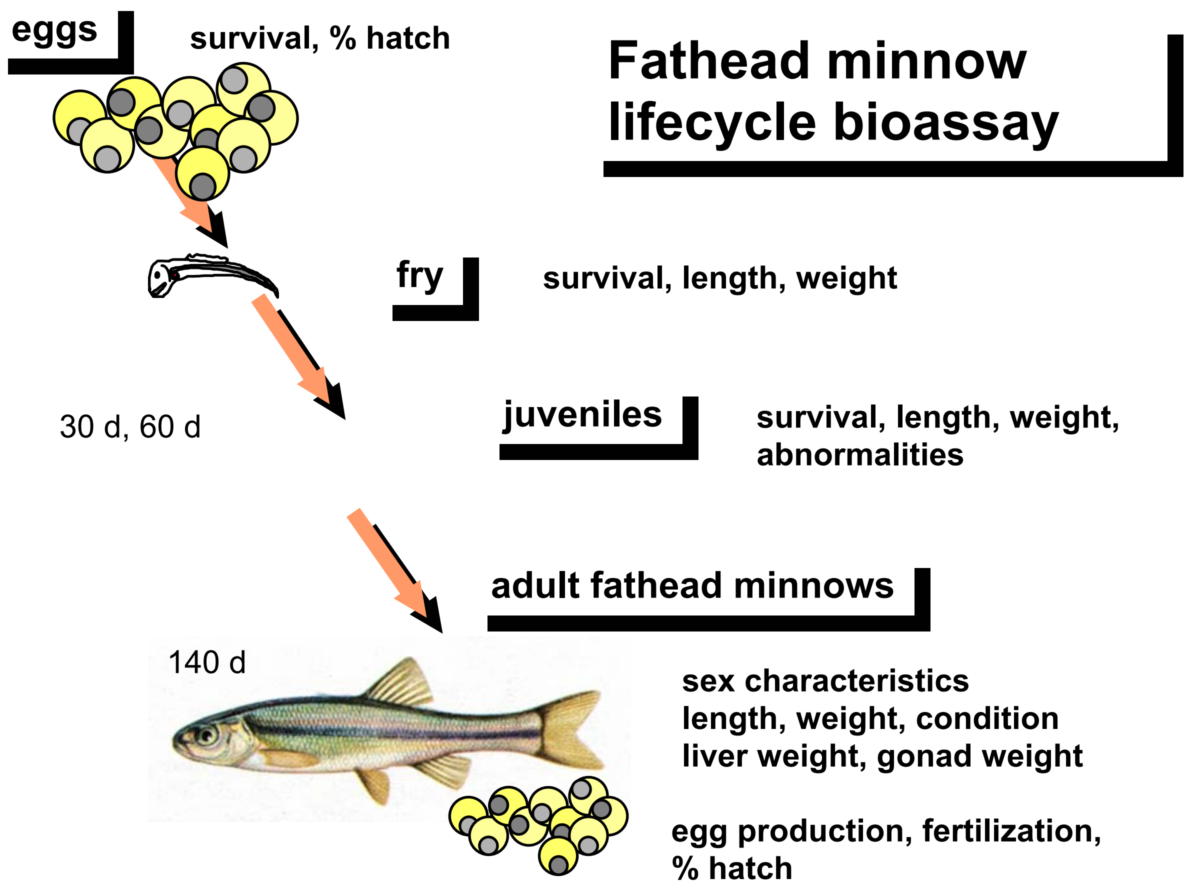
Long-term and lifecycle exposures of fish to PME have examined growth, maturation and reproduction (Figure 4). These long-term fish exposures are laborious, time consuming and expensive. However, they provide some of the most convincing evidence linking PME exposure to reproductive effects in fish. Similar to the effects seen wild fish exposed to PME, lifecycle exposures of small laboratory species have shown growth increases, alterations in sexual development, delays in time to first spawning, reductions in the numbers of eggs laid, and decreases in levels of sex steroids. Lifecycle studies often report changes in fish secondary sexual characteristics and decreased egg production as the most sensitive responses to PME.

Lifecycle studies have been conducted with many different fish species, such as fathead minnows, marine mummichogs, viviparous blenny (*Zoarces viviparus*), zebrafish (*Danio rerio*), Japanese medaka, mosquitofish (*Gambusia affinis*), guppies (*Poecilia reticulata*), or swordfish (*Xiphias gladius*). Requirements for lifecycle test species are that the fish are small, able to grow and breed in captivity, have a short generation time, and are sexually dimorphic. Because of relevance to the Canadian environment, lifecycle studies of have traditionally concentrated on fathead minnows and recently a few tests have been initiated with the marine mummichog.

Lifecycle exposures of fish to pulp mill effluents are conducted using a proportional diluter or series of pumps to deliver the desired concentration of pulp mill effluent to each exposure tank. Exposures begin with fertilized eggs, and fish are followed as they grow and mature. Growth, sex characteristics and breeding success are monitored. At the termination of the exposure (usually 4-6 months later) the adults are sampled and dissected to provide information on growth, liver weight, and gonad weight. Sometimes blood is sampled for measurement of sex steroids, although more commonly testes and ovaries are dissected and gonadal sex steroid production is assessed. In addition, various tissues can be preserved for histological examination.

Lifecycle assays were able to mimic many of the responses of wild fish exposed to PMEs. Lifecycle and long-term fish exposures have shown increased body size (length and weight) and increased liver size (Figure 4). Liver-somatic indices (LSIs) were increased in female and male fathead minnows exposed to bleached sulphite mill (BSM) effluent. Livers of fathead minnow from 50 % BSM effluent were two to three times the size of livers in control fish (Parrott & Wood, 2002). Enlarged livers and increased LSIs were seen in fathead minnows exposed for an entire lifecycle to bleached kraft mill effluent (NCASI, 2000). These changes are similar to the enlarged livers found in fish captured at sites downstream of pulp mill discharges (Hodson et al., 1992; Munkittrick et al., 1994a) (See *Field Effects and Mechanistic Studies*.)

Figure 4. Schematic of fathead minnow lifecycle bioassay and measureable endpoints.



Lifecycle exposures of fish to pulp mill effluents have shown changes in sexual development and decreases in or delays in the expression of secondary sex characteristics. Changes in expression of secondary sexual characteristics are a sensitive endpoint that responds to exposure to several endocrine-disrupting substances (EDS, such as ethinylestradiol and sewage effluent). Various types of EDS-related changes in secondary sex characteristics in fish exposed to PME have been reported: This includes delayed development of sex characteristics, de-masculinization of male fish, feminization of male fish, and masculinization of female fish.

Delayed development of sex characteristics and de-masculinization of male fish has been seen in laboratory fish and wild fish exposed to PMEs. Male fathead minnows exposed to BKME had a delay in appearance of secondary sex characteristics (Robinson, 1994). NCASI (2000) saw a delay in the development of secondary sex characteristics in fathead minnows exposed to a bleached kraft mill effluent. This is in agreement with the observation that wild white sucker (*Catostomus commersoni*) captured downstream of an Ontario BKME discharge showed decreased secondary sex characteristics (fewer nuptial tubercles in male fish; McMaster et al., 1991) (See *Field Studies and Mechanistic Effects*).

Masculinization of female fish and a shift of sex ratios towards more male fish have been seen after exposure to PMEs. Fathead minnows exposed to ≥ 2.5 % secondary-treated BKME for 275 d had increased male secondary sexual characteristics (Kovacs et al., 1995a). Masculinization of BKME-exposed female mosquitofish has been found over many years of study in Florida (Bortone et al., 1989; Drysdale & Bortone, 1989; Cody & Bortone, 1997; Ellis et al., 2000; Parks et al., 2001). The masculinization (increased length of specific anal fin rays) could be reproduced by exposure of mosquitofish to Mycobacterium degradation products of plant sterols (stigmastanol and β-sitosterol/campesterol mixtures) (Denton et al., 1985; Howell & Denton, 1989). Recently, these effects have been attributed to androgen agonists, as evidenced by binding of compounds to human androgen receptor and by activation of downstream luciferase genes in transfected CV-1 cells exposed to water from the river receiving the BKME (Parks et al., 2001). Jenkins et al. (2001) have isolated androstenedione in the most potent androgen receptor- (AR-) binding fractions of the Florida River that receives the paper mill effluent.

Masculinization of BKME-exposed fish has also been seen in other countries. There was an increase in the proportion of male zebrafish exposed to 50 % Swedish pulp mill effluent for 28 days (Örn et al., 2001). Increased proportions of male embryos/larvae were seen in viviparous eelpout (*Zoarces viviparus*) captured near a Swedish kraft pulp mill (Larsson et al., 2000). Masculinization of adult female mosquitofish was seen after 21 d exposures to 15 % secondary treated New Zealand pulp mill effluent. In addition, the New Zealand effluent-exposed masculinized females (with male-like gonopodia) showed male mating behaviours (Ellis et al., 2000).

Feminization of male fish has also been seen in lifecycle exposures of fish to pulp mill effluents. Fathead minnows exposed to BSM effluent from a Canadian mill for 5 months showed an increasing proportion of fish with ovaries (Parrott et al., 2003a). Exposure also resulted in premature development of ovipositors (an extension of the urogenital papillae of female fathead minnows used for laying eggs) in juvenile fathead minnows, and development of ovipositors in mature male fathead minnows (Parrott et al., 2003a, b). Pulp mill researchers in other countries have also seen apparent feminization: Zebrafish exposed to 50 % pulp mill effluent for 38 days had increased whole-body vitellogenin concentrations (Örn et al., 2001).

Complete and partial feminization of some male fish was seen in laboratory exposures of Chinook salmon (*Oncorhynchus tshawytscha*) fry after short exposures to very high concentrations of BKME. After a 29-d exposure to 100 % BC softwood BKME (and 75 d grow-out in clean water), 4 of 34 genetically male (with XY chromosomes) Chinook alevins (fry) were determined to be physiological females (Afonso et al., 2002). These XY males had gonads that resembled the developing ovaries of female salmon. In addition to the feminization, another 4 of the 34 XY male fish had intersex testes (testis-ova) and 13 of the 34 XY males had affected or underdeveloped testes (Afonso et al., 2002) as determined by histological examination. The intersex condition usually affected only one of the testes, with one normal testis and one testis containing developing oocytes among the testicular tissue. The physiological feminization of XY males was a persistent condition: 150 d after the 29-d exposure to 100 % BKME, two of fourteen genetic male salmon (sampled at 179 days post-hatch, dph) were found to be physiological females. The intersex condition and the delay in maturation or differentiation of the testes were transient conditions, seen only in fish at 103 days post hatch (dph), and not at 179 dph. Female Chinook salmon were unaffected by the treatments, although the authors caution that endpoints that may have shown endocrine disruption in females (such as increased plasma vitellogenin concentrations) were not measured in their study (Afonso et al., 2002).

Lifecycle exposures of fathead minnows to PMEs have usually found that egg production and time to first spawning are some of the most sensitive parameters studied (Robinson, 1994; NCASI, 1996; Kovacs et al., 1995a, 1996; Borton et al., 1997, 2000 and Parrott et al., 2003a,b). One of the first reported pulp mill fathead minnow lifecycle tests assessed secondary treated effluent from a bleached kraft mill (Robinson, 1994). After 6 months' exposure to 3 to 50 % BKME, fathead minnows had significantly reduced egg production and significantly delayed spawning compared to control fish. Male minnows had decreases in secondary sexual characteristics, but these were significant only at 50 % BKME (Robinson, 1994). This mill had historically been associated with alterations in fish growth and reproduction (See *Field Effects and Mechanistic Studies*).

Several types of PMEs reduce egg production and delay time to first spawning in fish. Studies of many bleached kraft mill effluents (Robinson, 1994; Kovacs et al., 1995a; Borton et al., 2000, 2001), several unbleached kraft mill effluents (Borton et al., 2000, 2001), one bleached sulphite mill effluent (Parrott et al., 2003a) have all found reduction in number of eggs produced, along with changes in secondary sex characteristics and sex steroid effects. Elemental chlorine-free (ECF) mills and unbleached mills also have final effluents that affect fish reproduction in lifecycle exposures. Borton et al. (2001) saw reproductive effects in fathead minnows exposed to ECF effluent, and to effluent from a kraft mill with no bleaching. Mill types that have not been associated with effluent-related reduction in spawning are a de-inking mill (producing tissue or towel from deinked office paper) and a thermomechanical mill (Kovacs et al., 1995a, Borton et al., 2000).

Researchers have tried to correlate reproductive endpoints in lifecycle exposures to other endpoints or biomarkers, to allow shorter meaningful tests to predict potential long-term impacts on reproduction. Egg production (measured as number of eggs per female per day) correlated most strongly (R^2=0.65) to time to first spawning, in a study of five lifecycle tests of four different PMEs (Borton et al., 1997). Other reproductive indicators (steroid hormone production by ovaries and testes, gonadosomatic index, egg hatch, egg diameter) and fish health and growth indicators (EROD, P4501A, liver-somatic index, and condition factor) did not correlate as well to egg production (Borton et al., 1997).

Egg production is one of the most sensitive endpoints studied in lifecycle tests of PMEs. Egg production was negatively affected at concentrations of effluent that were lower than (about half) the effluent concentrations that negatively impacted *in vitro* steroid production by excised ovaries and testes (Borton et al., 1997). This was similar to the finding of Robinson (1994) that steroid production in excised testes and ovaries was affected at BKM effluent concentrations two to three times those that negatively affected fecundity and egg production. Although EROD activity was measured in livers of fathead minnows, in all cases it was non-detectable (Borton et al., 1997). This is not surprising, as this species, similar to carp and goldfish, is relatively recalcitrant to MFO inducers. Borton et al. (1997) did see an increase in P4501A in fathead minnows, but it was not correlated to egg production, the most sensitive endpoint in their five lifecycle tests.

Lifecycle exposures of fathead minnows have been able to track improvements in final effluent quality. Fathead minnows exposed to ≥ 2.5 % secondary-treated BKME effluent for 275 d had lower egg production, and increased male secondary sexual characteristics (Kovacs et al., 1995a). A repeat of the lifecycle exposure after the BKM had installed process modifications and effluent treatment changes resulted in final effluent (tested up to 20 %) that did not alter reproduction or secondary sex characteristics of exposed fathead minnows (Kovacs et al., 1996).

The expansion of lifecycle tests, to multi-generation tests is laborious, expensive and time-consuming. However, it has been done for several pulp mill effluents. Multi-generation tests have been performed for some PMEs, and in general, most have found that effects between generations are fairly consistent, and there appear to be no increasing effects over generations. Borton et al. (2000) examined fathead minnow reproduction and health through four generations of exposure to various ECF and TCF PMEs in the US. Egg production was consistently decreased by exposure to both bleached and unbleached kraft effluents, but egg hatchability was not affected. Egg production was reduced to one third of that of control fish at BKME concentrations as low as 10 %.

Lifecycle tests with fathead minnows and long term fish exposures assessing fish (fathead minnow or marine mummichog) secondary sex characteristics, sex steroids, and reproduction are useful in the EEM program at sites where the adult fish survey requirements are difficult to achieve. In 2000, it became a new requirement in EEM to utilize laboratory and mesocosm approaches if adult fish capture or the field benthic survey was difficult because of tidal zones or other confounding factors (Environment Canada, 2000; Porter & Ellis, 2000). Lifecycle fish assays are also the definitive test for assessing the endocrine-disrupting or reproductive toxicity of compounds (such as pesticides and industrial chemicals) in the OECD and USEPA programs for screening and assessment of EDS (EDSTAC, 1998; OECD, 2000a,b; Parrott et al., 2001).

Sublethal tests were developed to mimic the responses seen in wild fish captured from PME receiving environments. These tests were used to assess rapid responses of fish, such as hepatic MFO induction, and steroid depression. Methods for steroids assessment improved, and techniques to measure short-term responses of fish gonadal steroid production were developed.

Several early full lifecycle reproductive tests showed that PME could alter reproduction in fish. As these lifecycle tests were lengthy and expensive to conduct, the focus then shifted to developing predictive shorter tests that could be used to screen effluents for their potential to cause long-term reproductive effects in fish. At the present time we are addressing this last issue: The development of cost- and time-effective laboratory assays that will predict reproductive changes in wild fish exposed to PMEs.

4.5 Conclusions

Laboratory tests have been used to answer many of the regulatory and research questions that have arisen related to the effects of pulp mill effluents on aquatic biota. Acute, short-term, laboratory tests have also been used to assess final effluents and to track changes in effluent quality over time. Short exposures of fish and invertebrates have clearly shown the improvement in final effluent quality through the installation of secondary treatment of effluent.

In an effort to predict impacts on wild fish, laboratory bioassays have been developed to examine the responses seen in fish captured in pulp mill receiving environments: MFO induction and steroid depression. Short-term exposures of laboratory fish to pulp mill effluents have been used to identify pulp mill-related chemicals that induce MFO and suppress steroids.

Sublethal tests were developed to mimic the responses seen in wild fish captured from PME receiving environments. These tests were used to assess rapid responses of fish, such as hepatic MFO induction, and steroid depression. Methods for steroids assessment improved, and techniques to measure short-term responses of fish gonadal steroid production were developed.

These short-term bioassays have enabled biological responses, such as MFO induction or steroid depression, to be traced to specific chemicals and components of pulp mill processes (such as plant compounds, and black liquors). Assessment of MFO induction and steroid reduction caused by PME has enabled comparison of types of mill processes, wood furnish, and effluent treatment. It appears that MFO induction and steroid depression are found at a variety of mills types, with and without chlorine bleaching, in hardwood and softwood pulping facilities, and before and after effluent treatment.

New genomic techniques have contributed to the ability of laboratory bioassays to detect changes associated with exposure to PMEs. The development of chromosomal markers to determine the genetic sex of some fish species has led to some novel findings of feminization of male fish after exposure to high concentrations of BKME. The pattern of gene expression in fish affected by PME is different from the gene expression pattern in fish exposed to estradiol. Improvements in these genetic technologies, and expansion of genetic markers for smaller, shorter-lived, laboratory test species, may lead to the development of shorter bioassays that are linked to reproductive effects.

Longer-term, lifecycle laboratory tests and mesocosm exposures have been used to assess the effects of chronic exposure to pulp mill effluents. These studies have demonstrated PME effects on growth enhancement of invertebrates and fish, liver enlargement, and decreases in gonad size and fecundity in several fish species. Laboratory tests have assessed pulp mill waste streams, and provided information to isolate and treat selected pulp mill wastewaters.

Several early full lifecycle reproductive tests showed that PME could alter reproduction in fish. As these lifecycle tests were lengthy and expensive to conduct, the focus then shifted to developing predictive shorter tests that could be used to screen effluents for their potential to cause long-term reproductive effects in fish. At the present time we are addressing this last issue: The development of cost- and time-effective laboratory assays that will predict reproductive changes in wild fish exposed to PMEs.

Long-term laboratory exposures of fish to pulp mill effluents have examined reproductive parameters (egg production, time to first spawn). Effects on growth, maturation and reproduction have been seen in final effluents from a variety of pulp mills, employing different pulping processes, and different bleaching chemicals. The most sensitive endpoint in these long term fish exposures is reproduction. This endpoint is biologically meaningful, but determining thresholds for effects requires lengthy and expensive tests. As the laboratory tests move forward into the next decade, attention will focus on the reproductive endpoints, and the possibility of shortening the bioassays while still maintaining sensitivity and biological relevance.

5. Characterization of Bioactive Chemicals

5.1 Summary

A large body of work has been completed in the last decade on investigating the sources and identities of bioactive substances present in final effluents from pulp and paper mills in Canada. Canadian research has focused on chemicals causing induction of detoxification enzymes and chemicals causing changes in the levels of reproductive hormones. Several things became apparent from the studies conducted during the early 1990s, including that i) it would be more difficult than originally assumed to identify the responsible chemicals, ii) there may be more than one chemical or group of chemicals responsible for impacts, and iii) the responsible chemicals were likely previously unidentified. Initial speculation about the role of chlorine bleaching and dioxins in the responses were addressed in the early 1990s, which determined that historical effects could only be partially attributed to dioxins and furans and that effects were not correlated with organochlorine discharges or AOX. By the mid 1990s, interest increased in trying to identify the characteristics of the chemicals responsible for induction of detoxification enzymes and the changes in steroid hormone levels. Research also expanded into the identification of individual waste stream sources of chemicals. The advent of suitable bioassays to drive investigative studies has afforded new opportunities to investigate chemicals affecting fish reproduction in the late 1990s to the present day.

From the work conducted to date it is apparent that the sources of bioactive substances originate from lignin degradation. This has the form of original digestion of wood furnish (black liquor, condensates derived from black liquor, black liquor carryover to bleach plant), and bleach plant effluents, which contain residual lignin removed during bleaching. There are indications that active substances can be recovered from wood directly. The best evidence now indicates that multiple compounds are functioning in multiple mechanisms affecting fish reproduction. Several compounds have been identified that cause elevated activity of detoxification enzymes but there remain multiple unidentified chemicals causing induction in final effluents. Evidence also indicates that continuous exposures are required to maintain the responses, suggesting the active substances are not persistent nor do they appreciably bioaccumulate. The releases of bioactive substances are not correlated with production or treatment processes.

5.2 Introduction

Pulp is produced from raw wood material and is the basic ingredient in the production of paper. Wood consists of cellulose, hemicellulose, lignin and wood extractives (McLeay & Associates, 1987). The objective of pulping is to separate and recover cellulose fibers from lignin and other wood constituents with maximum yield and minimum fiber degradation. Pulping is carried out by the application of chemical or mechanical energy to wood. Kraft pulping is the most common process employed globally, where wood chips are cooked with sodium hydroxide and sodium sulfide to dissolve lignin. After cooking, the pulp is washed and separated from residual cooking chemicals, known as weak black liquor. At bleached kraft mills, pulp is sent to a bleach plant where additional lignin is removed to achieve a desired brightness in the finished product; this is typically accomplished using chlorine and/or chlorine dioxide.

Large volumes of water are used in pulp production, which in turn generate significant effluent discharges to aquatic environments. A typical kraft mill discharges between 80,000 and 130,000 m^3/ day of effluent into surface waters. Effluents are a complex combination of waste streams produced in debarking, pulp washing, bleaching, and regeneration of cooking chemicals. Combined effluents are treated prior to release, typically in two stages. In primary treatment, suspended solids are removed in clarifiers and/or settling basins. In secondary treatment, microorganisms break down biodegradable material, which significantly improves effluent quality by reducing biochemical oxygen demand and reducing levels of organic compounds associated with toxicity (McLeay & Associates, 1987).

Final effluents are highly complex, consisting of hundreds of compounds, many of which are unknown. Wood-derived compounds present in effluents include wood extractives (terpenes, plant phytosterols), lignin, and lignin residuals. Characterization of effluent extractives has been confounded by the large amounts of high molecular weight lignin material present and variable effluent composition. Effluent composition is affected by mill-to-mill differences in process technology, process operation, differences in wood furnish, and chemical interactions among the different waste streams (McLeay & Associates, 1987).

In the last decade, pulp and paper mills in Canada have modernized their bleaching technologies and installed secondary effluent treatment systems to improve environmental performance. Despite these changes, effects on fish persist (Munkittrick et al., 1998, 2002a). Alterations in endocrine and reproductive function continue to be reported, including smaller gonad and egg size, increased age to maturation, decreased levels of reproductive steroid hormones, and altered expression of secondary sex characteristics (Leblanc et al*.,* 1997; Munkittrick et al., 1998; Dubé & MacLatchy, 2000a, 2001a).

The objective of this section is to provide an overview of studies conducted on the identities of bioactive substances present in effluents over the last decade, lessons learned and how research hypotheses changed as the issue developed.

5.3 Causal Investigations of Bioactive Substances

Numerous studies have attempted to address the paucity of information regarding the identities of bioactive substances present in final effluents. Although a variety of effects in fish have been associated with exposure to effluents in the field (See *Field Studies and Mechanistic Research*) and under controlled conditions in the laboratory or on-site (See *Development and Application of Bioassays*), the specific chemicals associated with the array of reproductive responses have proven extremely difficult to identify. Although effluent constituents such as β-sitosterol (MacLatchy & Van Der Kraak, 1995; Tremblay & Van Der Kraak, 1999), abietic acid, pinosylvin and betulin (Mellanen et al., 1996) have the potential to affect fish reproduction when tested individually, definitive cause and effect relationships have not been established because of effluent complexity, differences in species response patterns (e.g. between laboratory species and wild fish), and a lack of information on the mechanisms of action (Van Der Kraak et al., 1998).

Research in the area of compound identification has progressed on a number of fronts. Work in the early 1990s was focused on the identification of substances associated with cytochrome P4501AI induction (measured as ethoxyresorufin-O-deethylase or EROD activity; Figure 5). Work focused on this area because little was known about the characteristics of reproductive dysfunction in wild fish exposed to effluents and sources of acute toxicity had already been attributed to resin acids and guaiacols (Kringstad & Lindstrom, 1984), the levels of which are substantially reduced after secondary treatment.

Subsequent work from the mid 1990s to the present day have attempted to address the more complex issue of reproductive effects in wild fish (Figure 5), and those responses have now been conclusively demonstrated on a national scale through the Environmental Effects Monitoring (EEM) program (Lowell et al., 2003; Munkittrick et al., 2002a). Causal investigations of reproductive effects ranged from pure compound exposures, investigations of individual waste streams within the manufacturing process to Toxicity Identification Evaluations (TIEs). The TIE approach has been adopted by many researchers to isolate and identify chemicals associated with EROD induction. There has also been development of tests using fish and cell lines to drive chemical fractionations to investigate the causative agents (See *Development and Application of Bioassays*). Although ecologically relevant, reproductive dysfunction in wild fish has represented a much more difficult problem to address because the mechanisms are not understood. The responses have shown effects on gonad size, depressed levels of circulating steroids (Munkittrick et al., 1998), perturbations in the sex steroid biosynthesis pathway (McMaster et al., 1995a) and effects on gonadotropin production and peripheral sex steroid metabolism (Van Der Kraak et al., 1992), indicating multiple mechanisms and chemicals are involved. In the late 1990s development of suitable bioassays, such as fish-specific sex steroid receptor assays (Van Der Kraak & Biddiscombe, 1999; Wells & Van Der Kraak, 2000), life cycle tests (Parrott et al., 2003a) and short- term in vivo tests for steroid effects (Dube & MacLatchy, 2001a; Hewitt et al., 2002) has provided the opportunity to couple mechanistically linked endpoints to chemical fractionations in the investigation of the identities of the causative substances. This has led to the ability to formulate questions regarding the characteristics of bioactive substances, their relationship to production type and whether compounds associated with sex steroid depressions are related to other reproductive impacts (Figure 5).

Figure 5. Timeline and evolution of research questions of the isolation and characterization of bioactive chemicals in Canadian pulp mill effluents.

late 1970s	Phenols and guaiacols established as acutely toxic to fish
mid 1980s	Dioxins and furans found in effluents
1990	Do dioxins, AOX or specific chemicals correlate with effects observed in wild fish?
1991	Does secondary treatment reduce biochemical responses?
1992	What are the chemical characteristics of the responsible compounds?
1993	How do process changes affect chemistry and biological activity of effluents?
	Can we use reverse osmosis to isolate chemicals associated with MFO induction?
1994	What are the identities of chemicals that are Ah-Receptor ligands?
	What is the role of wood extractives and lignin degradation products in the responses?
1995	How do Ah-Receptor ligands differ between pulping processes?
	Can we isolate effects on steroid hormones to individual process streams?
	Can causal investigations be simplified by focusing chemical efforts on bioavailable compounds?
1996	Can we isolate and identify chemicals from process streams that cause MFO induction and steroid effects?
1997	Do effluent chemicals have the potential to affect sex steroid signaling and transport?
	Are sex steroid receptor ligands related to process type and treatment process?
1999	Can reverse osmosis be utilized to remove substances from waste streams that reduce steroid hormones?
2000	Do fish accumulate bioactive substances from non-kraft effluents?
	Can steroid hormone reducing substances be isolated from waste streams?
2001	Do fish accumulate bioactive substances under natural exposure conditions?
	Are biologically active compounds created in secondary treatment?
2002	Do extractives from different tree species have different biological activity?
	Can substances reducing steroid hormones be isolated in waste streams from other mills?

5.3.1 TIE studies

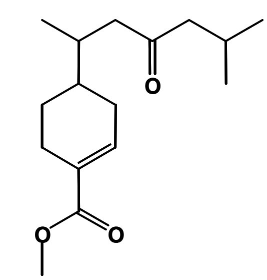
TIEs, developed by the US EPA, are an approach by which the active substances of interest in a complex matrix are characterized in three phases (USEPA, 1991, 1993a,b, 1997). The TIE approach is based on bioassay-directed chemical fractionations to identify unknowns and was developed for municipal sewage investigations in concert with Toxicity Reduction Evaluations (TREs) to ameliorate effluent acute and chronic toxicity. Phase I of a TIE involves determining the broad characteristics of the active agents through manipulations of the effluent. Phase II involves specific methods to isolate the active chemicals and propose structures for their identification. Phase III involves techniques to confirm that the proposed substances are in fact responsible for the observed toxicity.

Bioassay-directed effluent fractionations and TIE studies have been employed to isolate and characterize compounds associated with EROD induction in fish exposed to effluents (Burnison et al., 1996, 1999; Hewitt et al., 1996; Martel et al., 1997). In the early 1990s there was much speculation that polychlorinated dibenzo-dioxins and dibenzofurans (PCDD/DFs) present in final effluents as well as in biota from receiving environments were solely responsible for EROD induction observed in fish exposed to effluents (Figure 5). Although the physiological consequences of EROD induction were intensely debated (and still are), work was driven by the fact that enzyme induction represented exposure to compounds with the potential for dioxin-like toxicity. However, in the last decade an increasing body of evidence has associated non-dioxin classes of chemicals interacting with the Aryl Hydrocarbon Receptor (AhR). This evidence is collectively derived from i) the demonstration of EROD induction in fish after removal of defoaming agents from the manufacturing process that contained dioxin precursors, ii) the persistence in EROD induction following alterations in bleaching processes that do not promote polychlorinated dioxin formation, iii) transient EROD induction in fish upon cessation of exposure which is not characteristic of the sustained nature of induction associated with PCDD/DFs, iv) decreases in PCDD/DF levels in fish tissues collected following mill process changes, v) discrepancies between EROD induction and dioxin equivalents in fish measured in cell lines suggested that additional compounds may be involved (van den Heuvel et al., 1996) and vi) several studies which eventually associated EROD activity with non-dioxin effluent constituents. It is important to note that in the causal investigations conducted in the last decade, the total EROD activity of the effluents has not been accounted for and additional AhR ligands presently remain unidentified.

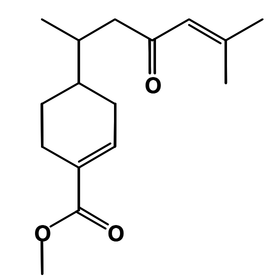
In the course of TIE studies conducted over the last decade, characteristics of the causative agents and selected individual compounds causing MFO induction have been identified (Figure 6). Hewitt et al. (1996) fractionated effluents before and after treatment and after a maintenance shutdown at a bleached kraft mill as one of the first studies to address the role of secondary treatment in affecting EROD activity. Laboratory rainbow trout were exposed to treated and untreated effluent, whole and filtered ($<1\mu m$) effluent, resuspended solids and two fractions of effluent that had been generated by nanofiltration. It was assumed that a comparison of relative EROD activity levels in the different effluents with chemical levels in the samples would provide insight into correlations of chemicals with the biological responses. For example, any chemicals found in whole effluent and in a fraction that was not associated with EROD activity could be eliminated as candidate inducers. Similarly, any chemical removed in a fraction or sample that retained EROD induction, could also be eliminated as a potential candidate. These analyses eliminated resin aids, fatty acids, bacterial fatty acids, terpenes, chlorophenolics, aliphatic alkanes, plant sterols and chlorinated dimethylsulphones as candidates. Although the comparisons found correlations of EROD activity with tetrachloroguaiacol, 4,5,6-trichlorotrimethoxybenzene and 2,4,6-trichloroanisole, only tetrachloroguaiacol was substantially above detection limits. Subsequent exposures confirmed that tetrachloroguaiacol did not cause induction, but Hewitt et al. (1996) concluded that the correlations might indicate the potential source of the compounds. Phenolics are among the major products of the oxidation of residual lignin during bleaching. When chlorine is used in bleaching, some of these phenolics may become chlorinated. The observed correlation with lignin-derived phenolics suggested that the inducers might also be lignin-derived.

Burnison et al. (1996) attempted to directly isolate chemicals inducing EROD activity in fish by following a classical TIE approach on final effluent from two bleached kraft mills located in Ontario. Using centrifugation, tangential flow filtration and C18 solid phase extraction (SPE), effluents after secondary treatment were investigated using a 4 d rainbow trout in vivo bioassay. It was determined that methanol extracts of particulates/colloidal material and SPE fractions contained active substances. Work focused on the particulate material and showed that activity could be isolated using HPLC. HPLC isolations determined that the active substances were present in a relatively nonpolar region of the chromatographic separation, with a logarithmic octanol/water partition coefficient (Kow) of 4.6-5.1. As a result of follow-up studies using rainbow trout exposures and incubations with a rat hepatic carconoma cell line (H4IIE) which directed HPLC fractionations of the methanol extract of the high molecular weight material, a chlorinated pterostilbene structure was postulated for an unknown compound strongly associated with induction (Figure 6; Burnison et al., 1999). This was significant in that it showed a natural product, modified in the bleach plant, was eliciting the biological response.

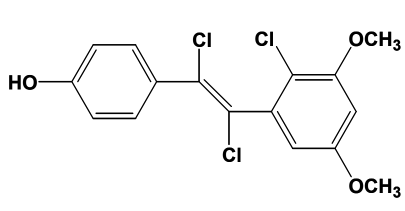
Figure 6: Non-dioxin AhR ligands identified in Canadian pulp mill effluents over the last decade.



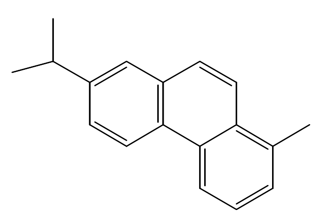
Juvabione



Dehydrojuvabione



Trichloropterostilbene



Retene

In a parallel line of work, Martel et al. (1997) determined the source and identities of two substances associated with induction present in the primary-treated effluent of a newsprint thermomechanical pulp (TMP) mill. To determine the sources of activity within the mill, rainbow trout were exposed static for 96 h to TMP condensate, deinking, and paper machine effluents and TMP whitewater and tested various process effluents sampled throughout the mill. Exposure concentrations were based on the flow of these process streams in relation to final effluent. Contaminated TMP steam condensates were identified as the major process source of EROD-inducing substances. Using conventional extraction, silica gel fractionation and preparative thin-layer chromatography (TLC) procedures, an EROD-inducing fraction was isolated. The major constituents were identified by gas chromatography/mass spectrometry (GC-MS) as juvabione, dehydrojuvabione, and manool, all naturally occurring extractives in balsam fir (*Abies balsamea*). After extraction and isolation from balsam fir and TMP condensates, trout exposed to juvabione and dehydrojuvabione exhibited significant hepatic EROD induction (Figure 6). This study further determined that secondary treatment in an activated sludge system effectively eliminated the EROD-inducing potential of the combined mill effluent consistent with a corresponding 90% reduction of both juvabione and dehydrojuvabione.

While the aforementioned study on TMP effluents appeared to have identified the source and identities of chemicals elevating EROD activity in fish, other researchers in Canada have investigated the causative agents at mills employing different pulping processes and wood species (Figure 5). One of the main reasons for doing so was the observation that EROD induction and reproductive dysfunction in wild fish persisted after installation of secondary treatment at a well studied bleached kraft mill (Munkittrick et al., 1992b).

Further studies of final effluents at other sites have proven problematic. Difficulties encountered include: i) fractionation experiments conducted on “grab” samples of effluent do not reflect temporal fluctuations in active chemicals, ii) toxicological potencies of effluent samples were influenced by sample handling and storage conditions, iii) the large amount of high molecular weight lignin material proved a significant interference when investigating low molecular weight extractives, iv) the complexity of low molecular weight effluent extractives and v) uncertainties regarding the bioavailability of identified bioactive components.

In the late 1990s, a different approach was adopted to address some of the obstacles associated with investigations of final effluents (Figure 5). Parrott et al. (2000c) used caged fish to investigate the uptake of AhR ligands from effluent from a bleached kraft mill. Ligands were recovered in two solvents used and non-dioxin ligands were found in tissue extracts using EROD induction in H4IIE cells as the indicator. This led to the development of a bioaccumulation model to investigate bioactive substances in complex mixtures. In these investigations the approach has been to focus on what is bioavailable to the organism by using controlled exposures to investigate bioactive substances in tissue residues (Hewitt et al., 2000a, b, 2003).

The bioaccumulation model was first developed at a bleached kraft mill that has been associated with reproductive dysfunction in wild fish since the late 1980s. Both naïve white sucker (*Catostomus commersoni*) and sucker collected adjacent to the effluent outfall were held in a concentrated effluent stream (50% v/v) for 4 d. Hepatic tissue extracts from exposed fish were fractionated according to lipophilicity using HPLC. Fractions with different octanol/water (K_{ow}) partition coefficients were tested for the presence of bioavailable chemicals that function as ligands for the

AhR in H4IIE cells, rainbow trout hepatic estrogen receptors (ER), goldfish testicular androgen receptors (AR) and goldfish sex steroid binding protein (SSBP). It was shown that fish rapidly accumulate multiple non-dioxin ligands for the AhR and fish sex steroid receptors after a 4 d exposure (Hewitt et al., 2000a, 2003). PCDD/DF equivalents measured by EROD activity in H4IIE cells and by high resolution GC-MS showed that in all fish historically exposed to effluent, the contributions to total toxic equivalents (TEQs) from TCDD was >80% and that in naïve fish held in effluent accumulated 1,2,3,7,8-pentachlorodibenzo-furan which accounted for a major portion of TEQ (Hewitt et al., 2000b). This study also showed that when fish normally residing in the effluent plume leave for a brief period to spawn in an uncontaminated stream, hepatic burdens of ligands for the AhR and sex steroid receptors decrease to the levels found in fish at reference locations. For wild populations historically exposed to bleached kraft mill effluent this suggests that a sustained exposure is required to maintain tissue concentrations. This study provided a plausible mechanistic linkage between uptake of ligands for sex steroid receptors and effects in wild fish and provided evidence of multiple effluent compounds functioning as ligands for multiple biological receptors.

A follow-up study at a bleached sulfite/groundwood mill was conducted to determine if the accumulation profiles of bioavailable substances were related to process type (Figure 5; Hewitt et al. 2003). White sucker again accumulated ligands for each receptor after 4 d exposure to effluent, and the pattern of accumulated substances was very similar to that previously obtained at the bleached kraft mill. Accumulated ligands were evaluated after exposure of fish to effluent for two different durations and following a depuration period. Hepatic EROD activity and whole-liver hormonal activity, measured as binding to SSBP, returned to background following 6 d depuration and were reduced but still significant after 12 d exposure to effluent. Whole-liver extract affinities for the AR were maintained after extended exposure and depuration, indicating the potential for AR ligands to bioaccumulate. By conducting these experiments at a bleached sulfite mill, this study provided further evidence that ligands for sex steroids and the AhR are associated with effects in wild fish.

5.3.2 Studies with pure compounds

In addition to systematically investigating effluents and waste streams using TIE approaches, researchers have followed hypotheses regarding effluent constituents with the potential to affect EROD activity or reproductive function in fish. A series of investigations have focused on retene (alkylated phenanthrene), an anaerobic degradation product of abietic and dehydroabietic acid, and its ability to induce EROD (Figure 6). Both parent chemicals are resin acids, common coniferous wood extractives, which are a family of acidic compounds commonly found in effluents. Resin acids are reduced, but not eliminated during effluent treatment and have historically been associated with effluent acute toxicity. Retene forms in treatment ponds and in sediments within waterways receiving pulp mill effluents. Retene has been found to cause elevated EROD activity in fish (Fragaoso et al., 1998), which was not sustained following transfer to clean water and is consistent with field observations (Munkittrick et al., 1992b). Spiking studies and studies with sediments collected in the vicinity of a bleached kraft mill contaminated with retene showed the bioavailability of retene and additional AhR ligands (Oikari et al., 2002). Further studies have shown that sediment retene is bioavailable and metabolites are detectable in bile of exposed fish (Billiard et al., 2000). Prolonged exposure results in a variety of dioxin-like toxicities to larval fish, including blue sac disease.

In the past six years, several researchers have hypothesized wood components are involved in reproductive responses (Figure 5). The focus on wood products has been based on the observation that reproductive responses in wild fish occur downstream of mills with very disparate processes (Munkittrick et al., 1998, 2002a), implying the effects are related to chemicals common to pulp mills, e.g., the wood furnish. This hypothesis is also supported by studies which have linked bioactivity in digest wastes, black liquor and bleachery process streams to lignin degradation products (see Process Streams and Conference Summaries below). When tested individually, β-sitosterol, the dominant plant sterol consistently measured in final effluents, exhibits estrogenic activity *in vitro* and can induce vitellogenin production and reduce plasma sex steroids *in vivo* (MacLatchy & Van Der Kraak, 1995; Tremblay & Van Der Kraak, 1999). Estrogenic potential has also been associated with abietic acid, pinosylvin and betulin (Melanen et al., 1996) and the flavonoid genistein (Pelissaro et al., 1991), which has recently been detected in bleached kraft effluent (Kiparissis et al., 2001). Studies with chemical recovery condensates have associated condensate components chemically resembling kraft lignin with testosterone depressions in fish (Hewitt et al., 2002). The confounding aspect of studies using pure compounds is that while effluent concentrations of many of these constituents are above threshold levels for effects, the same responses are not observed following effluent exposures. Suspected reasons for this involve response differences between laboratory and wild species, response pathways affected by other effluent constituents (Van Der Kraak et al., 1998) and interactions between effluent constituents.

In an extension of the work on sex steroid ligands accumulated by fish, in the late 1990s effluent samples were collected from 10 mills across Canada of various pulping and effluent treatment technologies to determine any relationship between pulping process, treatment systems and wood furnish (Figure 5; McMaster et al., 2002b). Ligands for fish sex steroid receptors were detected with higher activities observed for sex steroid binding protein and the androgen receptor, which reflects earlier observations in bioavailability studies (Hewitt et al., 2000a, b; 2002). Additional studies have shown the potential for effluents from several mills to exhibit antiestrogenic activity when extracts were co-incubated with estradiol in primary cultures of rainbow trout hepatocytes (Marlatt, 2003). Ligands for fish retinoic acid receptors have also been detected in effluent extracts from half of the mills surveyed, with high activity associated with effluents from thermomechanical facilities (Alsop et al., 2003). Active extracts were further partitioned into acidic and base-neutral fractions and it was found that most of the retinoic acid activity was associated with acidic components. At the present time it is not clear if ligands for retinoic acid receptors are also associated with depleted retinoid stores in fish collected from receiving environments but a mechanistic linkage has been established that can be addressed in future studies. In these series of effluent studies using in vitro tests there were no correlations with production type or treatment and it is unclear what effects, if any, conventional effluent treatment is having on levels of hormone ligands in effluent.

More recent studies on wood furnish have also attempted to address the question of whether bioactive substances can be isolated from wood chips prior to pulping (Figure 5). This question has arisen from previous work isolating natural products associated with EROD activity (Martel et al. 1997; Burnison et al. 1999) and early work which examined the effects of wood furnish on toxicity. When O'Connor et al. (1992) varied the wood furnish, simulated mechanical pulping effluents varied in acute and chronic toxicity. Chronic toxicity was measured using *Ceriodaphnia* reproduction and balsam fir effluents had the highest potency. Recent investigations have since determined that chemicals functioning as sex steroid ligands can be extracted using two different solvents from wood chips used in pulp manufacture (McMaster et al., 2002b). Ligands for fish retinoid receptors are also present in extracts of wood furnish (Alsop et al., 2003). Crude extracts were tested in these initial studies and the active compounds presently remain unidentified.

5.3.3 Process Stream Investigations

Beginning in the mid 1990s, several researchers have investigated individual waste streams within the papermaking process to determine the source(s) of EROD induction and compounds affecting steroid levels in fish (Figure 5). The principal challenge in these investigations has been to avoid acute toxicity associated with individual wastes, particularly those from the bleach plant. Researchers have primarily used in vivo fish tests in these investigations (See *Development and Application of Bioassays*) and the results will be summarized here in the context of what knowledge was gained on the sources of bioactive substances.

Black liquor was the subject of investigations involving EROD activity and hormonal endpoints. The pulping process digests lignin, the complex phenolic polymer that binds cellulose fibres together. The spent cooking liquor, known as black liquor, contains the degradation products of lignin and cellulose as well as wood extractives such as resin and fatty acids. Zacharewski et al. (1995) found that the methanol extract of black liquor particles and colloids > 0.1μ m from a bleached kraft mill contained AhR ligands which also displayed antiestrogenic effects via the AhR *in vitro*. Hodson et al. (1997) investigated the potential of black liquor from hardwood and softwood pulping at a bleached kraft mill to induce EROD activity in rainbow trout and found significant activity. A higher potency liquor was associated with alcohol digestion of wood chips as well as solvent extracts of wood.

EROD activity of final effluent from a bleached kraft mill with 60% ClO2 substitution was found to be reduced by > 90% using activated sludge treatment and an investigation within the mill showed bleach plant effluent to be the major contributor (Schnell et al., 2000). Follow-up work on bleach plants at two kraft mills in central Canada involved testing alternating chlorination (100% ClO_2) and alkaline extraction filtrates to determine which stages were associated with production of AhR ligands (Coakley et al., 2001). Filtrates were found to increase in potency through the stages of bleaching and the final bleaching stage was the most potent. Softwood filtrates also possessed more potency than hardwood filtrates, lending credence to the theory that the source of AhR ligands is ultimately related to wood furnish. The authors speculated that recycled wash water from the paper mill was a source of compounds in filtrates (Coakley et al., 2001).

As mentioned previously (5.3.1 TIE studies), process waste evaluations were used to identify candidate streams for TIE investigations at a thermomechanical mill (Martel et al., 1997). In that study, waste streams were evaluated flow-proportionally and steam condensates were ultimately identified as the major source of compounds causing elevated levels of EROD in rainbow trout. Specific chemicals, juvabione and dehydrojuvabione, were confirmed as AhR ligands (Martel et al., 1997).

In the late 1990s a systematic waste stream approach was applied to investigate sources of chemicals with the ability to affect sex steroid levels and/or production in fish at 3 other pulp mills. Extensive investigations on waste streams within two mills were conducted to determine their potential to elicit effects on circulating steroids in fish (Parrott et al., 2000a). Effluents before and after treatment were evaluated at a bleached kraft mill (18 streams) and a bleached sulfite mill (14 streams). In both cases, individual process wastes within the mill did not affect steroid levels or steroid production in goldfish (*Carassius auratus*) but final effluent from both mills after secondary treatment did cause significant steroid depressions. It is also interesting to note that final effluent from the bleached sulfite mill did not affect steroid levels after process changes that included i) increased ClO_2 substitution from 60-65%, ii) a reduction in solids losses from the bleach plant, iii) reduced liquor losses through spill management and iv) increased aeration within secondary treatment. It was unclear which of the process changes might be associated with the lack of steroid effects (Parrott et al., 2000a).

An extensive investigation was conducted at a bleached kraft mill in Saint John NB, which is one of a handful of pulp mills in Canada that does not employ secondary treatment. At the Saint John mill the study involved systematic exposures of mummichog (*Fundulus heteroclitus*) to 5 in-mill process wastes to determine the waste stream source(s) contributing to depressed sex steroids associated with exposure to final effluent. These exposures were first conducted in a field-based, mobile, artificial stream system (Dubé & MacLatchy, 2000a, b) and later confirmed with laboratory studies (Dubé & MacLatchy, 2001a). This work successfully resulted in the identification of chemical recovery condensates as a primary source of substances that depress circulating sex steroids in fish. Reverse osmosis treatment of condensates was also conclusively proven to remove the active substances prior to their re-use in brownstock washing and dilution before bleaching (Dube & MacLatchy, 2001b). RO treatment resulted in a non-acutely lethal final effluent and the sublethal toxicity of the final effluent was reduced in three different marine species (Dube & MacLatchy, 2000b).

Following the identification of condensates as a source of endocrine disruptors, TIE studies were initiated at the Saint John Mill to investigate the identities of the causative chemicals (Figure 5). Minimal high molecular weight material was found in the condensates, facilitating bioassay-directed fractionation studies (MacLatchy et al., 2001). Using steroid depressions in mummichogs, a two-step solid phase extraction (SPE) method was developed which completely recovers the active chemicals from the condensates in two fractions (Hewitt et al., 2002). Differences in the chemical composition and bioactivity of condensates generated during hardwood and softwood production were observed, which suggests a linkage to chemicals derived from wood furnish. Results also indicate that the responsible chemicals are polar, water soluble and bioavailable, which is supported by the steroid depressions that can be induced *in vivo* after a 7-d waterborne exposure. GC-MS profiles of both fractions revealed relatively simple mixtures of < 20 chemicals and the mass spectra of several unknowns appeared to be consistent with lignin degradation products (Hewitt et al., 2002). These findings are consistent with earlier studies which showed the onset of steroid perturbations in wild fish to be rapid and associated with multiple lesions in the steroid biosynthetic pathway (McMaster et al., 1995b).

5.4 *Characteristics of bioactive substances revealed during field and laboratory studies*

The ability of effluents to exert effects, independent of production and treatment type was identified in the mid 1990s (See *Field Studies and Mechanistic Research*) and more recently highlighted by the second cycle of EEM (Lowell et al., 2003). This suggests that at least similar compounds are being released by pulp mills at these sites and the compounds are not being eliminated during conventional effluent treatment. Waste stream studies have identified digester liquors (condensates, black liquor) and bleachery effluents as sources of bioactive substances. Collectively these observations suggest lignin as the origin of these compounds.

Although studies in the mid to late 1990s were able to isolate specific chemicals associated with MFO induction and in some cases able to assign chemical structures to AhR ligands in effluent (Figure 5; Burnison et al., 1999; Martel et al., 1997), unidentified chemicals are still elevating EROD activity in pulp mill effluents. Chemicals affecting reproduction in wild fish, specifically sex steroid levels, have proven more elusive. However, observations regarding the nature of the exposure and responses elicited during field and laboratory studies can provide clues as to the origin and properties of the responsible compounds. For example, there is evidence that compounds affecting steroid production in goldfish are produced during secondary treatment, as observed during waste stream exposures at a bleached sulfite mill and bleached kraft mill (Parrott et al., 2000a). There is also a body of evidence that shows that both EROD induction and affects on circulating steroids in fish require a sustained exposure to maintain the response and that once the exposure is removed both parameters recover (reviewed in Munkittrick et al., 1998; Van der Kraak et al., 1998). Other studies have shown that AhR ligands and ligands for sex steroids are accumulated within 4 d (Hewitt et al., 2000a,b) and are depurated within 6 d with the exception of ligands for the androgen receptor which are persistent in tissues (Hewitt et al., 2003). Compounds capable of reducing testosterone levels and gonadal production in chemical recovery condensates from a bleached kraft mill are readily bioavailable, polar and can elicit the response within 7 d (Hewitt et al., 2002).

The possibility of compounds acting synergistically or antagonistically complicates their identification as well as the mechanisms by which they exert their action. Recent studies with final effluents now indicates the potential for antiestrogenic activity associated with effluent from several process types, which may be associated with the lack of vitellogenin responses seen in wild fish and laboratory exposures (Marlatt, 2003).

5.5 *AOX: Regulation and relationship to effects*

As part of its regulatory program initiative, in order to determine what to do with organochlorine discharges under its legislation, the federal government conducted an assessment of effluents from mills using bleaching under the *Canadian Environmental Protection Act* (CEPA) in the mid 1990s. This was done to determine whether the effluents met the criteria for CEPA toxicity designation and to define how the releases should be addressed. The assessment concluded that the effluent met the toxicity criteria used under CEPA. However, the assessment also observed that the quality of the underlying science was insufficient to design regulations curtailing organochlorine discharges beyond what would be attained with the proposed dioxin-furan measures and the new FA regulations. Information deficiencies noted included knowledge of the make up of the organochlorine, since chlorine was present in a broad array of organic compounds with molecular weights from around 50 to multi thousands. Adding to this little was known about which compounds were responsible for the observed environmental effects.

In 1992, the Province of British Columbia modified the Pulp and Paper Mill Liquid Effluent Control Regulation (PMLECR) to require that the discharge of AOX from bleach plants be eliminated by the 31st of December, 2002. In December, 2001, the Minister of Water, Land and Air Protection, appointed a Scientific Advisory Panel to review the scientific basis of this requirement (Carey et al., 2002). The panel did not find evidence that the present level of AOX discharges from British Columbia's bleached kraft mills, and its one ammonia based sulphite mill, present a demonstrable risk to the ambient aquatic environment that could be attributed to AOX (Figure 5). The panel concluded that there was no evidence available to it at this time to indicate that further reductions of effluent AOX beyond that already achieved would result in any demonstrable environmental benefit. These findings were also supported by industry studies which found that AOX was not a good predictor of acute or chronic toxicity (O'Connor et al., 1993). While the panel concludes that the elimination of AOX is not a high priority as an environmental goal, it also concludes that it would be desirable to limit AOX discharges to levels comparable to those currently being discharged by the BC mills. Such a limit would make BC regulations comparable to those in most other pulp producing jurisdictions, and would ensure that the benefits achieved in recent years relating to reduced contamination of aquatic resources by pulp mill-related organochlorines are not lost.

5.6 Effluent and Receiving Environment Chemistry

In response to regulatory changes promulgated in the late 1980s and early 1990s, the greatest emphasis within the pulp and paper industry was to reduce the production and release of organochlorine compounds. Process changes, including oxygen delignification, chlorine dioxide bleaching, peroxide bleaching and improved brownstock washing have resulted in reduced organochlorine discharges, including total adsorbable organic halide (AOX), and also alterations in the composition of effluents (Servos, 1996). Oxygen delignification and improved brownstock washing have resulted in less residual lignin associated with the pulpstock prior to bleaching. This in turn means less bleaching is required to achieve the desired stock brightness and lower organochlorine content in bleaching effluents. The increased usage of chlorine dioxide in bleaching has further resulted in lower multiple chlorination of compounds such as chlorophenols, while having a lesser effect on the overall quantity of AOX discharges (Stromberg et al., 1996). In 1994, the use of chlorine dioxide in place of molecular chlorine, or elemental-chlorine-free (ECF) bleaching had become more common than the use of molecular chlorine. The significant differences in the chemistry of chlorine and chlorine dioxide provide a mechanistic basis for understanding the formation and character of chlorinated leaching by-products. Chlorine not only oxidizes lignin to form quinone or ring-opened structures, it also reacts through an electrophilic substitution mechanism to chlorinate aromatic rings and some aliphatic side chain groups. Chlorine dioxide reacts with the residual lignin only as an oxidant, forming ring-opened muconic acid (ester) structures (LaFleur, 1996).

The majority of AOX released from pulp mills is in the form of high molecular weight material (>1000Da), the composition of which varies according to pulping conditions. Questions about the significance of high molecular weight material formed in the bleaching process drove research into characterizing the structures of these materials in the early 1990s (LaFleur, 1996). Increasing the level of ClO_2 for Cl_2

increases the carbon to chlorine ratio, indicating a lower degree of chlorination. Numerous other studies determined that high molecular weight material after ClO_2 bleaching has a higher carboxylic acid content, and a higher phenolic content than residual lignin. A small proportion of the aromatic rings possess chlorine substitution, which should not lead to the formation of highly chlorinated persistent breakdown products from this material under environmental degradation processes. The breakdown of high molecular weight was considered by Millar et al. (1996) using fungal strains and sunlight. Both naturally occurring fungi and white-rot fungi were found to be capable of degrading high molecular weight fractions of effluent, which would reduce effluent colour and AOX. It was also observed that irradiation could result in the release of chlorinated guaiacols and vanillins.

In the early 1990s the majority of pulp mills across Canada had installed secondary treatment to comply with federal BOD and acute toxicity regulations. The effects on effluent compositions are complex and at that time were not well understood. It was recognized then that removal of compounds from effluent in biological systems is possible through microbial degradation, physio-chemical process such as adsorption, or air stripping. In some cases, microbial degradation may not be complete and it may also result in the formation and discharge of new compounds. It was further recognized that much of the work accomplished to date had been completed on the identification of compounds in process sewers, with comparatively less work on biologically treated effluents. This is partially due to the complexity of biologically treated effluents relative to process sewers and the fact that previous studies on process sewer compositions were the result of chlorine-based bleaching (LaFleur, 1996).

A review of the chemical properties of organics in final effluents in the mid 1990s reveals that the bioaccumulation of the majority of low molecular weight chemicals can be predicted with established relationships between bioconcentration factor and octanol-water partition coefficients (Muir & Servos, 1996). However, it was pointed out that the accumulation of many phenolics and neutrals will be overestimated because of metabolism. Many compounds attributed to pulp mill production were found to accumulate in wildlife in surveys conducted in British Columbia (Elliot et al., 1996). High concentrations of PCDD/DFs were found in eggs of various fish-eating birds species from the Strait of Georgia, with the highest concentrations near pulp mills. These results contrasted with concentrations found in the same species collected in the vicinity of pulp mills on the Atlantic Coast. Poor breeding success in blue herons (*Ardea herodias*) near a bleached kraft pulp mill in the Strait of Georgia was associated with a variety of effects, including EROD induction (Elliot et al., 1996). Principal component analysis of PCDD/DF concentrations in crab hepatopancreas had been used to distinguish individual mill sources and classify congener patterns according to chlorine bleaching and pentachlorophenol wood preservative sources (Yunker & Cretney, 1996). Since the late 1980s, the proportion of 2,3,7,8-chlorinated congeners and the overall PCDD/DF concentrations had begun to decrease near mill outfalls.

In the mid 1990s the concept of a minimal impact mill, based on a workshop conducted on the elimination of ECF and TCF-based bleachery effluents, was conceived. The workshop concluded that process development toward the minimum impact mill should begin by concentrating on minimizing releases from the pulping and recovery processes. The mimimum impact mill does not mean zero bleach plant effluent but one which maximizes pulp yield and produces quality products that are recyclable, maximizes the energy potential of bioamass, minimizes water consumption and wastes (Axegard et al., 1997).

Also in the mid 1990s, an ecological risk assessment of ECF effluents concluded that the risk of chlorinated compounds of concern had decreased dramatically following implementation of ClO_2 bleaching, which was supported by the re-opening of fisheries in the vicinities of pulp mills (Bright et al., 1997). Higher chlorinated phenols are not present in ECF effluents and the risk posed by lower chlorinated compounds is minimal. It was concluded that the compounds of concern are those that had received little attention thus far, the non-chlorinated compounds originating from pulping, rather than bleaching. The issue of the production of biologically active compounds of concern during secondary treatment, such as retene, was also raised.

The first work dealing with the ability of semi-permeable-membrane-devices (SPMDs) to concentrate biologically active compounds was conducted in the mid 1990s (Parrott et al., 1997a). Dialysates of SPMDs deployed in effluents from mills employing a variety of process types were able to elevate EROD activity in a fish hepatoma cell line. SPMDs also proved useful in the recovery of AhR ligands from in-mill process streams. In an extension of this work using fish caged in bleached kraft mill effluent, it was first reported that accumulation of AhR ligands could be measured (Figure 5; Parrott et al., 1997b). Male and female fish accumulated ligands for the AhR and these could be attributed to non-dioxin compounds following analysis of liver extract fractions generated by HPLC.

As part of the 1992 amendments to the Pulp and Paper Effluent Regulations under the Fisheries Act, every mill was required to conduct an Environmental Effects Monitoring (EEM) program on a three-year cycle basis. During the first cycle of the program in the mid 1990s, many mills experienced difficulties meeting the requirement to analyze for effluent tracers in fish. The objective behind tracer analysis is to provide collaborative evidence of exposure in the receiver, particularly where fish can move freely between sampling locations. An industry-government working group was established to examine the use and effectiveness of chemical tracers in exposure assessments and to develop recommendations for future cycles of EEM (Ali et al., 1997). Tracers commonly measured in the first EEM cycle were resin and fatty acids in fish bile and liver with additional analyses of chlorophenolics. It was determined that numerous factors affected the presence and concentrations of tracers in fish and that the concentrations of those tracers measured in the first cycle were highly variable. It was concluded that the inclusion of tracers in EEM is accepted in principle but additional round robin studies, quality control studies and field validation must be conducted in conjunction with additional EEM cycles to assess the effectiveness of the tracer program (Ali et al., 1997).

5.7 Conclusions

The installation of secondary treatment across Canada has substantially improved effluent quality through reductions of compounds causing acute toxicity. Organochlorine discharges and AOX have also been drastically reduced through implementation of additional process changes (such as 100% ClO_2 bleaching). However, effluents from pulp and paper mills continue to release bioactive substances into Canadian aquatic receiving environments that affect fish metabolism and reproductive performance. Causal investigations have provided insight into chemicals associated with EROD induction. The investigations have shown induction to be partially associated with wood constituents and remaining unidentified substances.

Addressing chemicals associated with reproductive effects in fish has proven to be a difficult challenge. Although certain wood components detected in final effluents have the potential to individually cause some of the observed effects, it is clear that other compounds functioning in additional mechanistic pathways are involved. The evidence to date shows that effects on sex steroids (and EROD induction) are the result of a rapid uptake of bioavailable compounds from effluent, that a sustained exposure is required to maintain the effect and that the responsible compounds do not appear to be bioaccumulative. The sources of bioactive substances in pulping processes appear to be derived from lignin degradation either from original pulp digestion or from residual lignin removal during bleaching. The identities of the responsible compounds have remained elusive, thereby impairing any evaluation of the effectiveness of secondary treatment and other process changes in the industry.

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