Theme Issue
Fate and Effects of Pulp and Paper Mill Effluents:
Select Papers from the 7th International Conference
Fredericton, New Brunswick, Canada
June 14-17, 2009
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- Impact of contaminants on aquatic ecosystems
- Contributions of pollutants from the gas and solid phases to aquatic systems
- Drinking water, wastewater, and stormwater treatment technologies and strategies
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THEME ISSUE

Fate and Effects of Pulp and Paper Mill Effluents:
Select Papers from the 7th International Conference
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June 14-17, 2009

Special Issue Editors

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In the late 1980s, worldwide attention was focused on the potential environmental impact of bleached pulp mill effluent after scientists in Sweden reported that fish collected near effluent discharges demonstrated changes in growth, carbohydrate metabolism, maturation, recruitment, mortality, and community structure (reviewed in Sodergren 1989). These studies were the impetus for the first conference on the Environmental Fate and Effects of Pulp and Paper Mill Effluents held in Saltsjöbaden, Sweden in 1991 (Sodergren 1992). Three years later, following implementation of new regulations and monitoring programs in several countries, and with corresponding implementation of new process and treatment technologies within the industry, the second conference was held in Vancouver, Canada (Servos et al. 1996). At this conference, there was a change in emphasis from studies examining chlorinated compounds to those focused on nonchlorinated compounds such as wood extractives and their metabolites. In 1997, the third international conference was held in Rotorua, New Zealand and concepts such as the minimum impact mill as well as Best Available Technology were discussed (Stuthridge et al. 2003). In 2000, from Helsinki Finland, the emphasis of the conference shifted once again, from a focus restricted to the aquatic environment, to a more broad-based environmental assessment that encompassed solid wastes and airborne emissions (Ruoppa et al. 2000). The fifth conference in this triennial series of meetings was held in Seattle, U.S.A., in 2003 and a special attempt was made to address the ecological significance of the remaining subtle effluent effects, and also to begin to understand the influence of discharges of well-treated effluents from modern process mills at the watershed level (Hall 2003). In an effort to bring together experts from the disciplines of forest industry wastewater treatment, the 2003 meeting was held jointly with the International Water Association's Symposium on Forest Industry Wastewaters. The success of this first joint meeting resulted in subsequent meetings being held jointly. In response to the significant growth of the industry in the Southern hemisphere, the 2006 conference was held in Vitória, Brazil (Furley et al. 2006). Holding the joint meeting in South America recognized the fact that the most modern, largest mills in the world are operating in South America. From an environmental standpoint, these mills pose interesting questions in that they pulp cultivated crops that are continuously replanted. However, little information was available regarding effluent effects on receiving environments.

The 7th International Conference on Fate and Effects of Pulp and Paper Mill Effluents returned to Canada and the 2009 meeting was held in Fredericton, New Brunswick with the 9th International Water Association Symposium (IWA) on Forest Industry Wastewaters. The conference was again dedicated to providing a forum where research on the environmental consequences of mill effluents was presented and discussed among interested stakeholders. There were a total of 18 countries represented at this international gathering, with 19 papers from the IWA Symposium being published in a 2010 issue of Water Science and Technology and 14 papers from the Fate and Effects Conference published in this Special Issue of the Water Quality Research Journal of Canada.

Changes to the global industry over the last 21 years of conferences have certainly been constant. Since the previous meeting in Brazil, the North American industry has been impacted significantly by reduced newsprint demand and currency fluctuations, resulting in a number of mill closures and corporate mergers. With this shift in production facilitating an already expanding South American industry, research on the fate and effects of mill effluents in South America has begun. To reflect this global change in the industry and new direction, we begin this Special Issue with a paper entitled, “Monitoring the Environmental Effects of Pulp Mill Discharges in Chilean Rivers: Lessons Learned and Challenges” by Gustavo Chiang and colleagues. Following this introductory paper, we move to a review of previous studies from the southern hemisphere entitled, “Summary of a Decade of

As some of the most significant alterations in fish downstream of mill effluents have included a reduced investment of energy in reproductive growth, the next nine papers all examine the effects of effluents on fish reproduction. The first of this series is based on a plenary presentation by Michael van den Heuvel that described the recent progress made in understanding the causes of endocrine disruption related to pulp and paper mill effluents. Following this paper are two papers from the United States by Noggle et al. that examine the masculinization of female fish exposed to mill effluents in Florida and the resulting influence on reproductive success. The next three papers discuss Canadian projects developed specifically to investigate the cause of reproductive disruption in fish. The first, by MacLatchy et al., presents a summary of 10 years of work on condensates at a mill in Eastern Canada identifying a treatment to remove the effects of effluents on steroid levels in laboratory-exposed fish. The next two papers, van den Heuvel et al. and Parrott et al. are the result of a collaborative project between the Canadian federal government, industry, and academia using data generated through the Canadian Environmental Effects Monitoring (EEM) Program in attempts to develop solutions for these effects. Part of this work is to investigate systematically which effects can be measured with short term exposures that are predictive of the lower reproductive investment observed in wild fish. This question was also addressed by Bosker et al. who examined the effects of a neutral sulfite semichemical pulp mill effluent that affects wild fish reproduction, using the same species in laboratory tests.

In Canada, the EEM program is a science-based monitoring tool designed to provide feedback on whether existing regulations are adequately protecting receiving environments. Munkittrick et al. present the most recent updates to the adult fish component of EEM, including species selection, critical effect sizes, and power analysis. Then, shifting back to New Zealand, Landman et al. present the evidence for improved fish health in two rivers following mill process modifications. The next paper reflects the truly global nature of the effects of mill effluents on different continents by examining effluent-related effects on phytoplankton and invertebrates in a Kenyan receiving environment. Kovacs et al. complete this series of papers by discussing what has been learned about effluent biotreatment in relation to environmental protection over the last 15 years and how these lessons can be applied to solving environmental impacts of mill effluents into the future. We end this special issue with a retrospective by Tim Hall and Dennis Borton, two U.S. scientists from the National Council for Air and Stream Improvement who have spent their careers studying the effects of mill effluents on the environment and reflect on what they have learned.

References


Monitoring of the Environmental Effects of Pulp Mill Discharges in Chilean Rivers: Lessons Learned and Challenges

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3 Faculty of Science, University of Ontario Institute of Technology, Oshawa, Ontario, Canada

Environmental monitoring activities in Chile are relatively new and have traditionally relied on physicochemical measurements. The pulp mill industry in Chile is highly competitive in the global market and several new large mills have recently opened. Early studies on fish in the receiving environments revealed lower species richness and an increase in the abundance of introduced species relative to native ones near pulp mill discharges. Even though changes were observed, their relationship with the discharges was unclear. Several difficulties related to small body sizes and the unavailability of basic biological data for native Chilean fish species were found during initial field studies. One of the main challenges is the standardization of monitoring methods (including fish species selection, sampling sizes, indicators, reference sites, etc.) and consensus about the responses that should be considered in a river monitoring program in the Chilean context. This paper summarizes major findings from a series of studies looking at impacts on fish at different levels of biological organization and the current approach used in Chile for monitoring impacts of pulp mill effluents on wild fish populations.

Key words: Chile, pulp mill, fish responses, biomonitoring, reproduction, monitoring design

Introduction

Pulp production began in Chile in the late 1950s and has grown exponentially during the 1990s and the present decade due to favourable conditions offered by the international market and certain competitive advantages in Chile (rapid growth of forest species used to produce pulp, lower labour costs, technological improvements, etc.). The Chilean pulp mill industry is presently within the top ten producers in the global market, annually producing more than 4.79 million tonnes of pulp. There are eight different mills in Chile (Table 1), and most mills discharge waste directly into freshwater river environments.

Three new mills built in the last 10 years were associated with several public controversies related to the sites chosen, an absence of information related to the potential impacts in Chilean ecosystems, and the potential impacts of development on other related economic activities (Parra and Acuña 2005). Public concerns raised by the new greenfield projects included some of the issues commonly addressed by the pulp mill industry worldwide, including the acute toxicity of wastewaters (Gaete et al. 2000; Mulso and Grandjean 2006), the presence of dioxins and furans in the effluents, and more recently the potential to cause reproductive problems in fish as reported in recent Canadian and Swedish studies. The concerns in Canada and Sweden led to the development of environmental effects monitoring (EEM) programs for pulp mills in both countries (Swedish EPA 1997; Environment Canada 2005), but no such monitoring programs existed in South America.

In Chile, the environmental regulatory process, administered by national and regional offices of the Comision Nacional de Medio Ambiente (CONAMA), addresses pulp mill discharges through separate emission regulations and environmental quality regulations. Both regulations have different spatial application: the emission regulations are at a national scale, while the environmental quality regulations have local specific applications (CONAMA 2004).

The concerns about pulp mills in Chile increased in profile in 2004 after a greenfield mill started to discharge to a river draining into a large wetland. The subsequent disappearance of a submerged aquatic macrophyte (considered a weed) was correlated with mortality in swans inhabiting the wetland. Although no cause-effect relationships have been established, a series of scientific research publications pointed out several potential causes, such as natural changes (Marin et al. 2009), the impact of heavy metals (Jaramillo 2005), and potential effects of ultraviolet radiation (Ramírez et al. 2006). However, from a toxicological point of view, no specific chemical cause-effect relationships were established (Palma et al. 2008). In this context, during the environmental impact assessment for that new mill in Chile, a monitoring program for dioxins and furans in the effluent and the receiving environment was required by the regulatory agency (CONAMA). Our previous experience in the evaluation of those chemicals in a different Chilean watershed impacted by pulp mill discharges was unable to correlate concentrations in fish liver—total TEQs [toxic equivalents] for PCDD/Fs [polychlorinated...
dibenzo-p-dioxins/dibenzofurans] and coplanar PCBs [polychlorinated biphenyls]; 2.87–6.45—with biomarkers of contamination (EROD [ethoxyresorufin-O-deethylase] induction levels preimpacted < impacted < postimpacted; <1 to >70 pmol/min/mg of protein) (Orrego et al. 2005). This was in agreement with the expected reduced dioxin and furan concentrations in effluents from mills using the elemental chlorine free process (Servos et al. 2003), which was the case for all new industries in Chile.

This important issue raised the public concern of pulp mill development not only in Chile, but also in other South American countries, and this affected other greenfield projects. The local authorities requested a series of complementary studies, and the industry developed voluntary monitoring programs to address the issues. From our point of view, this fact totally changed the relationship between the industry and the community, and a series of commitments were signed by the industry in order to answer the public concerns. Given the extensive international work, the emerging national issues, and the uniqueness of Chilean ecosystems, the aim of this paper was to use studies conducted to date to develop an adequate strategy that could be used in Chilean pulp mill monitoring programs in order to satisfy the specific demands of all stakeholders involved.

### Strategies for Evaluating Environmental Effects

There are a variety of approaches used in environmental monitoring programs that include fish. When designing a monitoring program, the advantages and disadvantages of the different responses and species that can be used should be considered. While many programs within the United States focus on monitoring at the fish community level (Yoder and Rankin 1998), in Canada they focus on the population level (Environment Canada 2005), and the EEM program in Sweden requires information at the biochemical, individual, population, and community levels (Swedish EPA 1997). According to our experience in Chilean environments, a useful approach uses the responses at different levels of biological organization. In this sense, the use of individual organisms is essential as it allows follow-up studies to develop at both lower organizational levels to understand mechanisms (e.g., tissue, physiological, biochemical), and at upper organizational levels to understand ecological relevance (e.g., population, community) (Munkittrick et al. 2000).

### Basic Design Following the International Experience

The most common monitoring design for evaluating new discharges in Chile has been the classical “Before-After Control Impact (BACI) design,” which is based on evaluating the potential impacts prior to project establishment (also called baseline evaluation) as well as after. Attempts are made to increase the number of reference sites to avoid misinterpretation, as recommended by Keough and Mapstone (1997) for Australian EEM programs. Existing mills have been examined using a control impact or gradient design when predevelopment conditions were not known.

Fish have played an increasing role in monitoring programs dedicated to detecting and assessing the potential impacts of industrial effluents in industrialized countries such as Canada (Munkittrick 2004; Lowell et al. 2005; McMaster et al. 2006), the United States (Sepúlveda et al. 2002; Theodorakis et al. 2006; Yeom and

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### TABLE 1. Pulp production in Chile

<table>
<thead>
<tr>
<th>Name</th>
<th>Region</th>
<th>Company</th>
<th>Kraft type</th>
<th>Tonnes per year (1,000)</th>
<th>Receiving environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Licancel</td>
<td>Maule</td>
<td>Arauco</td>
<td>BSKP/BEKP</td>
<td>145</td>
<td>Mataquito River</td>
</tr>
<tr>
<td>Celco</td>
<td>Maule</td>
<td>Arauco</td>
<td>UKP</td>
<td>350</td>
<td>Sea</td>
</tr>
<tr>
<td>Laja</td>
<td>Biobio</td>
<td>CMPC</td>
<td>BSKP</td>
<td>260</td>
<td>Biobio River</td>
</tr>
<tr>
<td>Arauco</td>
<td>Biobio</td>
<td>Arauco</td>
<td>UKP</td>
<td>80</td>
<td>Biobio River</td>
</tr>
<tr>
<td>Santa Fe</td>
<td>Biobio</td>
<td>Arauco</td>
<td>BSKP/BEKP</td>
<td>260</td>
<td>Sea (Arauco Gulf)</td>
</tr>
<tr>
<td>Santa Fe Línea II</td>
<td>Biobio</td>
<td>CMPC</td>
<td>BEKP</td>
<td>380</td>
<td>Biobio River</td>
</tr>
<tr>
<td>Pacífico</td>
<td>Araucania</td>
<td>CMPC</td>
<td>BSKP</td>
<td>500</td>
<td>Biobio River</td>
</tr>
<tr>
<td>Valdivia</td>
<td>Los Ríos</td>
<td>Arauco</td>
<td>BSKP/BEKP</td>
<td>685</td>
<td>Cruces River</td>
</tr>
<tr>
<td>Nueva Aldea d</td>
<td>Biobio</td>
<td>Arauco</td>
<td>BSKP/BEKP</td>
<td>856</td>
<td>Itata River</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>4,791</strong></td>
<td></td>
</tr>
</tbody>
</table>

*BSKP: bleached softwood kraft pulp; BEKP: bleached eucalyptus kraft pulp; UKP: unbleached kraft pulp.

Empresas CMPC S.A.

Started August 2006.

Started September 2006.
Adams 2007), Sweden (Larsson et al. 2000; Sandström and Neuman 2003), Finland (Donald 2003; Karels and Oikari 2000), and New Zealand (van den Heuvel et al. 2007). However, the use of environmental monitoring programs within a governmental framework is not frequently used. Indeed, the present review found that biological monitoring programs existed in only three of the four countries that are the largest producers of pulp and paper worldwide (Table 2), establishing high cost effectiveness as the primary objective when designing a program to monitor effects in fish.

In Sweden, the Swedish EPA (1997) and Sandström and Neuman (2003) established a national pulp and paper effluent monitoring program to assess the potential changes in a top-down approach by measuring biological parameters at multiple biological levels to discriminate enrichment effects from toxicity/hormonal disturbance. Monitoring parameters included the community structure of fish, and population to individual and biochemical responses in one of two species (Zoarces viviparus or Perca fluviatilis), depending on the receiving body of the discharge. The scale and effects were not defined specifically, but varied according to the receiving environment characteristics. In Canada, the critical effect size was based on historical data review (Munkittrick et al. 2009), but in Sweden, this size was determined by a multistakeholder consensus review (government, industry, and academia). This process had initially set the level of change thought to be unacceptable (Table 2). Any changes detected later in monitoring were evaluated on an acceptable to unacceptable scale, according to responses in various functional groups, from population characteristics to physiological functions and contaminants in fish. Since these criteria were determined by collective bargaining, their environmental relevance remained unclear.

In comparison, the EEM program in Canada was based on a legal mandate under the Fisheries Act to protect fish, fish habitat, and the use of fisheries resources. This program evaluates population and individual level responses in at least two fish species on a cyclical basis (Environment Canada 2005). The results of the first cycle of monitoring were reviewed and discussed by a panel of scientists and government and industry representatives. This panel ultimately defined a 25% difference in size of the gonads and liver, and a 10% difference for condition factor between fish. This program included a sampling design, with highly comparable results ($\alpha = 0.05$ and $\beta = 0.20$) for pulp and paper mills at the national level (Table 2). More recently, a decision has been made to set $\alpha = \beta$, and a review of international approaches to setting critical effects sizes has validated the effects sizes chosen (Munkittrick et al. 2009).

A different philosophy is used for monitoring pulp mills in the United States where there are few legislated field monitoring programs for fish, and the U.S. EPA (1998) supports the development and use of BAT (best available technology) in their pulp and paper industry. This approach only provides limited physical and chemical parameter information on effluent discharge

<table>
<thead>
<tr>
<th>Country</th>
<th>Monitoring strategies</th>
<th>Endpoints</th>
<th>Effect magnitude</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>MBACI-SCI; Environmental differences; Site specific monitoring design.</td>
<td>No specific sentinel species or biological endpoints due to ‘high variability of the receiving systems in Australia.’</td>
<td>Scalable magnitude from a multiple stakeholder negotiation; Type I and Type II error rate (0.5); 50–100% of change unacceptable.</td>
<td>Keough and Mapstone 1997</td>
</tr>
<tr>
<td>Canada</td>
<td>Legal mandate.</td>
<td>Individual level responses in at least 2 fish species, data distribution between populations exposed and nonexposed to effluents</td>
<td>Gonad and liver size $&lt;$25%; Condition factor $&lt;$10%.</td>
<td>Environment Canada 2005; Lowell et al. 2005</td>
</tr>
<tr>
<td>Sweden</td>
<td>Stakeholder negotiation; Nationwide monitoring program.</td>
<td>“Top-down” strategy; Multiple endpoints, from community structure to biochemical indicators in two sentinel species: <em>Perca fluviatilis</em> and <em>Zoarces viviparous</em>.</td>
<td>Division of endpoints in functional groups establishing: unacceptable perturbation of function, unacceptable perturbation in fish health, evident risk to population, and need of confirmation of results.</td>
<td>Swedish EPA 1997; Sandström and Neuman 2003</td>
</tr>
<tr>
<td>U.S.A.</td>
<td>No biological monitoring strategy, EPA supports the development of BAT.</td>
<td>Nonbiological endpoints; Concentration thresholds for certain harmful components in effluents.</td>
<td>Emission standards below detection levels</td>
<td>U.S. EPA 1998</td>
</tr>
</tbody>
</table>

*MBACI-SCI: Multiple Before-After Control Impact-Scalable Decision criteria.

*BAT: best available technology.*
in water environments (Table 2). However, there have been a number of studies that have used fish to assess the effect of some pulp mill effluents (Sepúlveda et al. 2002; Theodorakis et al. 2006).

Pulp mill activity has expanded in Brazil and Uruguay, and is predicted to increase rapidly in the future throughout South America (Villalonga 2006). Uruguayan studies associated with a new pulp mill have adopted the Canadian EEM model to evaluate the effects of effluent discharges, in addition to the traditional physicochemical monitoring of water quality in a highly controversial development (Joutsenvirta and Vaara 2009). However, since this is a preliminary evaluation, no major conclusions for this monitoring strategy for this newly developed industry have been made.

In the design of monitoring programs, sample size calculations are possible given knowledge of the standard deviation of the endpoint, acceptable type I and type II error rates, and preset critical effect sizes for the endpoints of choice (Munktittrick et al. 2009). There are recommendations for setting type I and II error rates in Canada (Environment Canada 2005), and in Australia, they should be set at a 0.5 ratio (Keough and Mapstone 1997). Although biological monitoring programs exist in Australia, Canada, and Sweden, they all have a different background design frameworks (Table 2).

The Triad Approach for Chilean Environments.

From a Chilean standpoint, a good starting point for the evaluation of effects is a “triad approach” (Chapman and Hollert 2006), which considers: 1) monitoring of field populations, 2) semicontrolled experiments using standard species as bioindicators (i.e., caging the same fish species at different sites with similar environments), and 3) laboratory bioassays using both effluent and sediment samples. Each approach answers different questions but looks for the same responses in order to determine if the effects observed in the field are correlated to effluent exposure. The fish monitoring program helps with understanding the general state of health of the wild fish populations. In some locations however it is only possible to conduct manipulative experiments controlling exposure time and location. This type of experiment can also be affected by other factors such as vandalism and cage destruction, as previously reported (Oikari 2006; Orrego et al. 2006). While field observations provide valuable information, their interpretative power can be reduced because of confounding factors. Thus, laboratory-specific effluent exposure and the evaluation of receiving environment matrices emerge as an appropriate complementary strategy to assess the specific effect with a greater capacity to establish the causal relationships.

Multivariate analysis of laboratory results, field evaluation experiments, and chemical evaluations should help establish specific relationships that can help define environmental thresholds to evaluate the impact of industrial activities on aquatic systems. This strategy can provide important information to be used to improve environmental management and regulation of aquatic ecosystems, particularly in Chile, where threatened and endangered species are often involved. Much of the research conducted by our research group has been designed to accomplish these objectives (Orrego 2006; Orrego et al. 2005, 2006, 2009a). However, implementation has been complicated due to difficulties when conducting the experiments, scarce access to funding, copyright issues associated with proprietary data in private contracting research, and challenges in communication with private companies. This has made it difficult to establish a significant environmental threshold for the pulp and paper mill industries that discharge into Chilean environments.

Research from a recent experiment compared lab exposure of pulp mill effluent to the results observed in caging experiments using the control impact design. Results showed a correlation between lab exposure and field caging studies (Fig. 1). The analysis allowed the identity of two different indicators in rainbow trout—plasma vitellogenin (Vtg) production and gonadosomatic index (GSI)—that were associated with a gradient in dilution in both cases. A clear distinction could be drawn
from the experimental data that helped establish potential thresholds that must be verified in the field (when the same species is used in the monitoring), helping to define the effluent's adverse effects in the receiving environment. First, Vtg induction was induced 12-fold on average, which correlated with an effluent concentration of 35% in caging experiments in the postimpact zone. These exposures were also correlated with increases in gonad size of 35%, which represents a threshold for the caged rainbow trout. However, further research is required to define a general threshold for native fish species at this site as well as at other sites using different industrial processes.


The design of EEM programs for pulp mill effluents must be cost effective; this is especially important in developing countries compared with other countries with existing regulations. Hewitt et al. (2008) pointed out that most of the published effects of pulp mill effluent identify alterations in fish reproductive and metabolic rates, which have also been identified by our group in Chile under laboratory and field conditions using rainbow trout (Onchorynchus mykiss) (Orrego 2006; Orrego et al. 2005, 2006), as well as at the molecular level (Chamorro et al. 2010). The main effects associated with Chilean pulp and paper mill effluent exposure to date are consistently described as estrogenic responses, which differ from the mainly androgenic and antiestrogenic effects reported in Canada and New Zealand (Orrego et al. 2009a). Recently, the same estrogenic effects seen in rainbow trout have also been observed in native Chilean fish species, with a possible stimulation of the reproductive system resulting in the consequent disruption of normal sexual maturity. These changes have resulted mainly in a stimulation of in vitro estradiol production by the gonads, induced maturation during recrudescence, and smaller female gonads during spawning seasons, all disrupting the population dynamics of native fish populations exposed to effluents (Chiang et al. in press—a). These reproductive endpoints (molecular, cellular, individual, and population dynamics) must form part of any specific Chilean monitoring program.

The adoption of parameters and strategies from other programs already in place in other parts of the world is also recommended, but should be adapted to the reality of local systems (Munkittrick et al. 2009). Consequently, using a strategy similar to that of the EEM program in Canada (Environment Canada 2005) appears to be adequate to evaluate reproductive patterns in fish populations, but it should be complemented with the laboratory and semicontrolled field evaluations already developed by our research group.

Critical effect sizes for gonad and liver sizes (2.5%) as well as condition factor (10%) have been evaluated in native Chilean fish species (Chiang et al. in press–b), although these parameters have not been correlated with the changes detected under laboratory conditions using introduced species. The design of cost-effective monitoring programs should assess these reproductive abnormalities and metabolic capacities. It is important to incorporate assessment tools that link the information obtained in the triad approach without compromising accuracy, effectiveness, and reproducibility for detecting possible alterations (Table 3).

Monitoring Challenges in Chilean Rivers

Chilean rivers are characterized by short distances, steep slopes, and low biodiversity (Habit et al. 2006a). These rivers face natural changes in chemical and physical stressors along the river, and have adapted over time to such drastic changes. Additionally, the low biodiversity in fish fauna, the sometimes narrow geographic range of unique species, and the widespread introduction of exotic species has affected the availability of native fish. Thus, these characteristics are important to consider during the implementation of monitoring programs to assess impacts of industrial and urban discharges in Chilean rivers.

Since native species have only been studied in the last 25 years, their biology is in general poorly understood. Consequently, the principal challenge when developing standardized monitoring programs in Chilean environments is a better understanding of the biology of native fish species. Unless we can document the natural variability within defined values, we cannot compare responses of native fish populations exposed to pulp mill effluents. Additionally, these gaps in basic biological data for Chilean fish increases the difficulty of evaluating responses at a very low level of biological organization, such as biochemical or molecular biomarkers (e.g., EROD, Vtg). Therefore, detection limits and methodologies have to be improved for native species, and must be calibrated with other species’ responses.

Another factor that has to be considered when designing a pulp mill monitoring program is the chemical complexity of pulp and paper mill effluent (Hewitt et al. 2008). The chemicals present relate to the wood source material, the industrial process used, and the type of effluent treatment employed. The environmental fate of these chemicals and potential impacts will also depend on the receiving water morphology and physical and chemical characteristics, as well as the species’ sensitivity. The selection of a fish species for the monitoring program is also a complex task since there is no one single criterion for the selection. Under our strategic approach, several factors should be considered when making this choice, especially those related to industry and the local environment (Fig 2).
The selection of the spatiotemporal scale of analysis is also highly relevant. Changes in community-based measures are useful to establish the ecosystem’s condition and to reveal damage, especially in a highly endemic environment such as found in Chilean river systems (Habit et al. 2006a, 2006b). However, these indicators are difficult to use for establishing potential causal relationships, and understanding the causes is crucial for potential restoration/protection of that aquatic ecosystem. Furthermore, the community structure is influenced by processes operating at different spatial and temporal scales, which makes it more difficult to correlate with the effects of pulp and paper effluents and to implement standard process and/or effluent treatment changes to prevent deleterious effects (Maltby and Burton 2006). Long-term studies in the United States have shown a healthy and diverse community structure in aquatic systems that have received a variety of industrial discharges for more than 100 years (Burton and Hall 2009), and have been unable to show any change at the community level by using biotic condition indices (Hilsenhoff Biotic Index) in a multivariate analysis (Flinders et al. 2009). Impacts at lower levels may not translate to the community level, or the variability at the community level between seasons and sites may be unable to show potential impacts (Orrego et al. 2009b). The use of population parameters, individual responses, and tissue level effects (physiological and biochemical variables) have been demonstrated to be suitable tools that provide evidence on the effects of industrial discharges. Such studies have been done in independent monitoring programs in several countries: Canada (Munkittrick 2004; Lowell et al. 2005), the United States (Sepúlveda et al. 2002; Theodorakis et al. 2006), Sweden (Larsson et al. 2000; Sandström and Neuman 2003), Finland (Donald 2003; Karels and Oikari 2000), New Zealand (van den Heuvel et al. 2007), and Chile (Orrego et al. 2005, 2006a&b). Responses at these levels happen sooner than community-level responses, are more easily reversible, and are easier to link to effluent exposure. In this way, our approach supports identifying the ecological significance of the impact as well as providing a warning of environmental risk for the fish fauna by establishing causal relationships, while at the same time providing a protective value to monitoring programs (Fig 3). The advantages of a combined approach are obvious in terms of obtaining the benefits of ecologically relevant changes, with changes in reproductive performance, energy storage (i.e., condition factor and lipids), and survival as indicators of ecosystem quality (Munkittrick et al. 2000), and the short-term specific biochemical changes as early indicators of the causal variables (Orrego et al. 2009a, 2009b). However, the identification of an indicator that links both levels is certainly necessary, where the potential evaluation of a reproductive biomarker, such as Vtg in native species, appears to be the key. That objective is possible in Chile using the alkali-labile protein phosphorous method (Kramer et al. 1998; Pollino et al. 2009), which can be applied for several species.

### TABLE 3. Biological endpoints in fish for effects monitoring program

<table>
<thead>
<tr>
<th>Population characteristics &amp; physiological functions</th>
<th>Biological endpoints</th>
<th>Use</th>
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</thead>
<tbody>
<tr>
<td>Abundance (capture per unit effort)</td>
<td>Wild fish monitoring</td>
<td></td>
</tr>
<tr>
<td>Recruitment (length frequency)</td>
<td>Wild fish monitoring</td>
<td></td>
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<tr>
<td>Age at length</td>
<td>Wild fish monitoring</td>
<td></td>
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<tr>
<td>Reproduction</td>
<td></td>
<td></td>
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<tr>
<td>Gonadosomatic index (GSI)</td>
<td>Mechanistic studies and/or wild fish monitoring / in situ bioassays / laboratory bioassays</td>
<td></td>
</tr>
<tr>
<td>Egg production</td>
<td>Mechanistic studies and/or wild fish monitoring / in situ bioassays / laboratory bioassays</td>
<td></td>
</tr>
<tr>
<td>Gonad histology</td>
<td>Mechanistic studies and/or wild fish monitoring / in situ bioassays / laboratory bioassays (need to develop biological data for native species, use of data from exotic fish spp)</td>
<td></td>
</tr>
<tr>
<td>Sex steroids (production or circulating)</td>
<td>Mechanistic studies, unless a major analytical development occurs in Chile</td>
<td></td>
</tr>
<tr>
<td>Plasmatic vitellogenin</td>
<td>Mechanistic studies and/or wild fish monitoring / in situ bioassays / laboratory bioassays (need to develop biological data for native species, use of data from exotic fish spp.)</td>
<td></td>
</tr>
<tr>
<td>Use and energy storage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition factor (k)</td>
<td>Mechanistic studies and/or wild fish monitoring / in situ bioassays / laboratory bioassays</td>
<td></td>
</tr>
<tr>
<td>Liver somatic index (LSI)</td>
<td>Mechanistic studies and/or wild fish monitoring / in situ bioassays / laboratory bioassays</td>
<td></td>
</tr>
<tr>
<td>Hepatic function &amp; detoxification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EROD activity</td>
<td>Mechanistic studies and/or wild fish monitoring / in situ bioassays / laboratory bioassays</td>
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</table>
The use of sentinel species gives us useful information on the state of the environment and the modulation of abiotic variables on the responses or effects observed in individuals captured (Munkittrick et al. 2000; van der Oost et al. 2003), but several stressors could alter certain biological endpoints outside of natural variability. The caging bioassays offer advantages in terms of the ability to use them in aquatic systems or seasons when you cannot perform successful environmental monitoring, and allows controlled effluent exposures. These in situ bioassays are more realistic than the traditional laboratory bioassays because they include natural exposure routes, compensation mechanisms, and environmental factors, and also allow the control of fish size, age, reproductive status, sex, number of exposed individuals, spatial gradients, replicates, and time of exposure (Oikari 2006; Orrego et al. 2006; Crane et al. 2007). When using exotic species under semicontrolled conditions, extrapolation to the population level should be avoided (Sпромберг and Meador 2005). In contrast, the use of native species for in situ tests allows direct extrapolation to higher levels (Chiang et al. unpublished data). These can be
verified directly through biological monitoring in the same area (Baird et al. 2007). Additionally, the use of native species minimizes stress during exposure because they are adapted to spatial and temporal fluctuations of environmental variables that occur in the exposed area (e.g., temperature, pH, conductivity, dissolved oxygen) (McWilliam and Baird 2002, Baird et al. 2007).

Since the upper trophic levels in the river systems reflect the energy flow through the ecosystem, and knowledge of the natural variability of the responses tested is required, reference sites are important to consider. The assumption is that the sentinel species used reflects factors or characteristics of the site where they are collected (Bowman and Somers 2005). Habitat changes that result in a change in the energy flow at this level vary their performance characteristics, and therefore it is assumed that changes in performance reflect impacts (Munkittrick et al. 1992). Moreover, one of the primary considerations is the residence time and exposure of the individual or population to the effluent. Large fish present a clear advantage when collecting the samples required for biochemical analysis, but they may have high mobility which can take them outside the effluent exposure area (Katano et al. 2006). In contrast, smaller fish have proved very helpful as a model for assessing the effects of pulp mill effluents in river systems (reviewed in Munkittrick et al. 2002), mainly due to their lower mobility and relatively short time to reach sexual maturity. Therefore, monitoring programs using small fish can integrate responses over one or more generations more easily (Ankley and Johnson 2004), although there can be problems with sample requirements for biochemical analyses.

Finally, laboratory tests help to isolate the effects of the compound or group of compounds and allows additional control of the size, age, reproductive status, sex, and number of exposed individuals, as well as time of exposure and doses, all adding to a reduction in variability (Crane et al. 2007; Orrego et al. 2009a). However, there are a number of artefacts in this type of analysis associated with the low environmental realism with laboratory bioassays (Baird et al. 2007; Crane et al. 2007). It is possible that two or more species can have the same physiological response and sensitivity to a chemical substance, but the life history strategies (i.e., differences in sexual maturation time, survival rates at different stages of life cycle, reproductive frequency) may alter the significance of the responses and consequences to the population (Schaaf et al. 1993; Caswell 2001 in Spromberg and Meador 2005). Consequently, there are questions about the ecological significance of laboratory studies since they offer limited ability to extrapolate to field conditions (Crane et al. 2007). Their use should be restricted only to the different lines of evidence used in the evaluation of effects of chemical stressors in the aquatic environment or to identify compounds

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**Fig. 3.** Ideal responses of biological endpoints without stress (natural variability range) and exposed to one or multiple stressors (Modified from van der Oost et al. 2003).

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responsible for the alterations observed in the field, and to examine mechanisms associated with demographic characteristics and toxicological endpoints (Spromberg and Meador 2005). This reduces the uncertainty in risk assessments, and the order of significance for an accurate determination of the dominant stressor and its ecological significance should be laboratory studies < in situ bioassays < field studies (Burton et al. 2003; Oikari 2006).

Data interpretation is a key issue since there is currently no agreement in considering how big the deviation of certain endpoints should be to consider the change an effect, and what should be done when an effect is observed. The standardization of the monitoring programs should discuss these issues since the definition of an effect could be based on: 1) statistical considerations, based on the variability of the endpoints used, or 2) quantal responses expressed as a percentage of change (Munkittrick et al. 2009). A good starting point could be to establish a threshold from laboratory experiments, and use these thresholds as surrogates to set up when an observed response during the pulp mill effluent monitoring is considered an adverse effect. It is important to evaluate the relationships between effects observed in laboratory bioassays and in controlled experiments in the field. Even though differences in magnitude between laboratory and semi-field experiments can be observed, a good correlation between responses at different levels of biological organization will help to establish critical correlations in the responses.

Although endpoints measured at the individual level seem to be appropriate for monitoring purposes, some biomarker responses, such as production or plasma levels of hormones, could be considered in studies where reproductive impairments are observed. This would also be the case with molecular endpoints such as detoxification enzymes, plasma vitellogenin production, and or gonadal production of hormones. For monitoring purposes, according to the international experience, suborganism level endpoints, including condition factor, relative organ sizes (liver somatic index, gonadosomatic index), gonadal histology, and egg production, may be used, with two or more species being used (Table 3). Clearly, a lot of work will need to be performed to be able to extrapolate results from the laboratory to the field in order to establish quantitative relationships and reduce the confounding factors related to the variability within species and between species. Ideally, the work should be conducted with the same species, although this is not an easy task.

**Conclusions**

It is obvious that programs based only on chemical or biological endpoints do not sufficiently ensure proper environmental protection. Indeed, a program that incorporates both chemical and biological monitoring strategies, such as the experiences in Canada, Sweden, and Australia, are a step ahead. These approaches have been shown to be widely beneficial and accepted not only by the government but also by the public and the industry. Even though reproductive effects have been consistently identified in the Canadian program, the chemical compounds causing those effects still need to be identified (Hewitt et al. 2008). This fact reinforces the idea that good monitoring programs must consider complementary responses in the biota and chemical evaluations.

Even though the Chilean industry has put a lot of effort into implementing the best available technologies and improving the final effluent treatment systems, reproductive effects continue to be observed in fish in our receiving environments. Further research is required to better understand the significance of these effects, especially at the fish population and community levels. It is clear that in the next few years, the dialogue between stakeholders needs to improve, and more independent research should be performed in order to address these important issues. Fortunately, some of the industry has been proactive, but more work is needed to improve the collaboration and compromise between industries, researchers, and the government in order to develop specific environmental monitoring strategies that should be seen as opportunities to improve industrial and environmental sustainability.

Important challenges remain. Strong monitoring programs require a better agreement between regulatory authorities, academia, and industry. These programs need to consider economic and scientifically relevant criteria. Stakeholders need to understand that more monitoring requirements (e.g., physicochemical measurements) do not necessarily lead to better environmental protection, and that the public discussions initiated by the Chilean National Commission on the Environment (the future Ministry of the Environment) should continue. As scientists, we must understand that sometimes the discussion is not limited only to technical aspects, and an effort needs to be made to find the right technical answers which all stakeholders can accept.

In environmental impact evaluations of future and existing pulp and paper mill industrial activity in Chile, it must be recognized that pulp and paper mills are highly dynamic (as reflected in production increases and technology changes) and that the Chilean industry is moving away from discharging into rivers with plans to discharge into the sea. Thus, there is the need to begin analyzing effects in the marine environment. A logical issue to address is the relevance of the effects observed in rivers to these in the marine environments. The Canadian EEM program has identified difficulties in studying marine environments, including tidal action, floating effluent plumes, and movement of species (Boyd et al. 2002). Additionally, further study is required to determine if the new treatment technologies are able to remove the chemicals causing reproductive impacts. To respond to these critical questions, we need to advance the monitoring...
requirements by ensuring that adequate sample sizes are collected, the right endpoints are measured, and the analytical capabilities are improved. Additionally, since many Chilean species are listed as “protected,” we need to make progress in nonlethal sampling strategies and nondestructive techniques (such as population size structure dynamics/recruitment and molecular methods for using mucus or blood where possible) for analyzing reproductive and metabolic endpoints. All these advancements should help us determine whether or not the existing effluent and environmental quality standards effectively protect the environment for us and for future generations.

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References


Orrego R. 2006. Efectos de las descargas de efluentes de celulosa y papel en peces en el río Biobío, Chile central: uso de biomarcadores fisiológicos, bioquímicos y reproductivos. Tesis de Doctorado, Universidad de Concepción, Concepción, Chile. 153 pp.


Summary of a Decade of Research on the Effects of a New Zealand Pulp and Paper Mill on Reproduction in Fishes

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The effluent of the Tasman pulp and paper mill (Kawerau, New Zealand) has been intensively studied for its effects on the health of fishes between 1998 and present. This review summarizes peer-reviewed scientific literature on the reproductive effects of the Tasman Mill effluent on fishes. In the 1990s there was an emerging body of literature from around the world showing that exposure to pulp and paper effluent could cause subtle reproductive alterations in exposed fishes. Locally, the Tarawera River had proved to be a difficult environment to conduct field studies. To overcome some of the difficulties with studying fish populations in the Tarawera River, initial studies on the reproductive health of fishes were focused on mesocosm and laboratory bioassays. During the later part of this period of study, wild fish population sampling was conducted in-river to assess the cumulative impact of multiple discharges. The initial mesocosm studies were conducted with rainbow trout exposures over an entire reproductive development cycle. The Tasman Mill effluent was initially observed to cause reductions in gonad size in females corresponding with lower circulating sex steroid hormones and reduced egg and larval sizes. This result was not observed again in the two subsequent long-term exposures conducted after 2001. Laboratory studies initially found the effluent to have a masculinizing effect on female mosquitofish (Gambusia affinis). This mosquitofish masculinization response disappeared after 2001 and was also not seen in effluent-exposed wild populations. Upstream and downstream populations of the native common bully (Gobiomorphus cotidianus) showed different reproductive timing, and investigation revealed that genetic differences were a potential reason for these differences. Subsequent investigation compared the Tarawera River bully to genetically similar Rangitiki River bully and found no evidence of reproductive alterations. The entire body of published data was assessed with regards to changes at the mill and chemical profiles of the effluent. It was evident that continuing effort on the part of the mill has resulted in gradual improvement in effluent quality over the duration of the studies. However, the disappearance of reproductive effects as assessed by multiple bioassays corresponds to one major change; screen room closure in the pulp mill. This change would have resulted in wood extractives being shunted from the treatment system to the recovery boiler, resulting in a net reduction in compounds derived from wood.

Key words: fish, reproduction, pulp and paper, endocrine disruption, mesocosm, laboratory

Introduction

International research on the environmental impacts of pulp and paper mill effluents on biota in receiving environments in the 1980s and 1990s led to the design of a research program to examine the effects of the Tasman Mill effluent in Kawerau, New Zealand, which discharges into the Tarawera River in the Bay of Plenty Region. The state of knowledge in the mid 1990s suggested that with secondary treatment being the rule rather than the exception, acute lethality was no longer a major environmental concern. This was certainly the case with the Tasman Mill effluent that had received secondary treatment since 1972. Organochlorines were no longer a major scientific concern due to the switch to chlorine dioxide as a bleaching agent. However, the complete transition to elemental chlorine free bleaching at the Tasman Mill was not completed until 1998. The removal of acute effluent effects led to observations of more subtle physiological effects in fishes. In particular, the published data that suggested effluent could lead to reproductive dysfunction in fishes was a serious, though controversial concern (Van Der Kraak et al. 1992; Munkittrick et al. 1994, 1997; Kovacs et al. 1997). These observations came precisely at the time when the phenomenon of “endocrine disruption” exploded globally as a public issue. As many of the observed impacts were based on field studies with wild-captured fish, there was considerable controversy as to the validity of those studies with regards to comparable habitat, genetics, fish exposure, reference site choice, and other confounding variables that are a fact of field studies.

In addition to the global state of knowledge on the fate and effects of pulp and paper effluents, there were a number of factors specific to the Tarawera River that influenced the experimental routes taken. At the time, most wild fish surveys were conducted with large-bodied fishes. Eel are the only native large-bodied fish in the Tarawera River and their life cycle prohibits the
study of the effects of effluent on their reproductive development. An in situ study had been conducted with the native shortfin eel (*Anguilla australis*) that showed elevated hepatic monoxygenase (MFO) enzymes (Jones et al. 1995). A single study had been conducted with rainbow trout (*Oncorhynchus mykiss*), but the period of residency of this species in the mainstem of the river was questionable and reproductive parameters were not examined (Donald 2003). Many fish species in the downstream Tarawera were highly migratory, making them poor monitoring species, and there was a general lack of basic biological and physiological knowledge of other potential monitoring species.

The downstream Tarawera River, once part of a large wetland on the Rangitiki plains, was heavily channelized in the early 1900s, and the high flow velocity in combination with a rolling pumice bed make the river poor fish habitat. The altered downstream habitat led to relatively low fish abundance and difficulties in sampling. The Tarawera River had multiple effluent inputs including two pulp mills, sewage, stormwater, and geothermal (sewage effluent is no longer discharged to the river). Thus, it would be difficult to tease apart the relative impacts of those effluent inputs from the Tasman Mill effluent, which was the furthest downstream.

In the mid 1990s environmental staff at the Tasman Mill made the decision that environmental effects research at the mill should ultimately enter the public domain through peer-reviewed research. This was a reflection of confidence in the effluent quality at the mill, a culture of ongoing improvement, and the wish of staff that research should hold as much credibility to stakeholders as possible. The studies of the Tasman Mill effluent continued for over a decade until the present day. This review will summarize the results of this body of research and draw conclusions as to the overall effects of Tasman Mill effluent on the reproductive physiology of fishes.

### Research Program Design

The Tasman Mill is located adjacent to the Tarawera River in the Bay of Plenty Region of the North Island, New Zealand (Fig. 1). The Tasman Mill uses both kraft and thermomechanical pulping (TMP) processes. When the initial studies were conducted, the mill produced 760 and 1,010 air dried tonnes per day of kraft and TMP pulp, respectively. Presently, kraft production is at 820 air dried tonnes per day and the mill produces a similar quantity of newsprint. Initially, the mill furnish was largely softwood (*Pinus radiata*) with the occasional pulping of *Eucalyptus* sp., while at present the mill uses about one-third eucalypt with the remainder of the pulping furnish being pine (all of the newsprint furnish is pine). Kraft pulp was chlorine bleached at either of two bleach plants with sodium hypochlorite (HH, now discontinued) or chlorine dioxide (DEopDnD or DEopPD - with the latter being currently used) (D = chlorine dioxide; Eop= alkaline extraction reinforced with oxygen and hydrogen peroxide; P= peroxide; Dn = chlorine dioxide reinforced with nitrogen compounds). The Tasman Mill has been elemental chlorine free since April, 1998. The TMP effluent was pretreated in-mill using an aerobic moving bed bioreactor from the onset of the studies until 2006 when the bioreactor operation was ceased. This pretreated effluent, and the effluent from the remainder of the mill operations, was collected into a single drain and after screening and settling, was treated in an aerated lagoon system with a 5- to 6-d retention time prior to release to the Tarawera River. The Tasman Mill has had secondary treatment since 1972. The mean total effluent flow of the Tasman Mill was approximately 180,000 m³/d, which had been reduced to 130,000 m³/d over the time span of these studies. The dilution of this effluent in the Tarawera River ranged between 5 and 12% (vol/vol) over the duration of the studies (currently approximately 7.5%).

The circumstances at the Tasman mill in the mid-1990s described above contributed to the building of a research program on the health of fishes exposed to mill effluent (Fig. 2). The program was designed not only to assess potential toxicity of the Tasman Mill effluent, but also to answer a number of more general scientific questions regarding the fate and effects of pulp and paper mill effluents.

The difficulties with using wild fishes and with multiple confounding effluents in the Tarawera River influenced a research program that would initially depend heavily on the use of artificial exposure systems, or mesocosms. The mesocosm research after 1998 focused primarily on the reproductive health of rainbow trout. Because facilities were constructed on site at the Tasman Mill, effluent could be provided on a regular basis, thus
Reproductive Impacts of a New Zealand Pulp Mill Effluent

Fig. 2. Overview of experimental program for determining the biological effects of Tasman Mill effluent on fishes.

averaging out the week-to-week variability in effluent strength that occurs at any pulp mill. These studies maintained the maximum environmental relevance with natural river temperature and ambient photoperiod while controlling for confounding variables, particularly energy (food) intake, a major factor in wild fish studies.

The mesocosm studies were supported by laboratory bioassays and in vitro bioassay approaches that were more focused on determining potential causative agents in the effluents. In particular, the international scientific focus at the time (which has continued to the present day) was on estrogens, and, to a much lesser extent, androgens in the environment. While such bioassays lacked environmental relevance in terms of determining potential population level impacts, they are more predictive to the presence of specific causative agents of reproductive dysfunction. As alterations to growth, reproduction, and survival are the only three means by which populations can be impacted, the overall product of the program provided a comprehensive picture with endpoints relative to those factors.

Finally, with an improved understanding of the direct biological effects of the effluent, a change in research focus was required to attempt to delineate the effects of Tasman effluent in the receiving environment relative to the complexities of cumulative environmental factors in the river. This review summarizes the three major areas of research, 1) mesocosms studies, 2) laboratory bioassays, and 3) field studies, in three major sections. Specifics of the methodologies used are given in each section. The report then synthesizes the major findings in light of their chronological order as it relates to effluent chemistry and in-mill changes.

Tasman Mesocosm Studies

In 1998, an experimental mesocosm facility, composed of six 12,000-L epoxy-coated fibreglass tanks, was constructed at the Tasman Mill primarily for rainbow trout effluent exposure experiments. Full strength (100%) effluent was transported by tanker truck and stored in an 80,000-L concrete holding reservoir adjacent to the experimental tanks prior to dilution with upstream Tarawera River water. The mean measured dilution of effluent in the exposure tanks averaged approximately 12% for the series of experiments conducted over a period of four years. Trout were held under ambient light and temperature conditions, and each was implanted with an individually numbered T-bar type tag to facilitate growth measurements.

The mesocosm studies were comprised of five separate trout experiments (van den Heuvel and Ellis 2002; van den Heuvel et al. 2002, 2004, 2008; Ellis et al. 2005). Experiments were designed to test a number of different hypotheses (for example the effects of exposure timing and ration). However, there is sufficient commonality in design to compare data across experiments in order to examine temporal trends. The exposure density was between 63 to 80 trout per 12,000-L tank for all experiments. Trout were fed commercial salmon food and there was at least one group of trout with a ration level of 0.7% of wet body weight per day in every experiment that was used for all year-to-year comparisons. All experiments to examine adult trout reproductive physiology were initiated in September with the exception of the first experiment in 1999 that started in January and was terminated just prior to the natural spawning period (this sampling date varied between late April and early June). Gonad growth in this strain of trout was observed to begin in late December. All experiments were initiated with age two-plus trout, with the termination of the experiment occurring near age three. However, in the case of the experiment terminated in 2003, trout were age four and had been exposed to effluent for nearly two years. Most of the experiments included a component of egg fertilization post adult exposure.

Adult Rainbow Trout Results

There was no indication of elevated rainbow trout mortality due to effluent exposure in any of the long-term mesocosm experiments. Rainbow trout exposures showed that a reduction in ovary size was only observed in the experiment that ended in the year 2000 (van den Heuvel and Ellis 2002). Reductions in ovary size were due to smaller ovarian follicles rather than due to fewer follicles. During the 2000 experiment, both a two-month and an eight-month exposure were carried out, but only the eight-month exposure showed reproductive effects. The 1999 experiment was only two months in duration, identical to the two month exposure completed in 2000.
These shorter-term exposures were both carried out during the latter part of vitellogenesis and are consistent in the lack of impacts observed at this time. The females in 2000 showed significant correlations of gonadosomatic indices (GSI) (measured upon termination of the experiment) with estradiol, testosterone, and vitellogenin (vtg) measured at several intervals during the experiment from the initiation of vitellogenesis to prespawning (van den Heuvel and Ellis 2002). Steroid hormones were measured for all of the experiments, however in many cases, they were measured only at the termination of the experiment. Steroid hormones were found to be of limited value during the period immediately preceding spawning due to the rapid changes that occur in steroid levels at this time. The 2000 experiment demonstrated that steroids showed a better relationship to GSI during mid and particularly early vitellogenesis.

Rainbow trout consistently showed no changes in liver size or in condition factor over the various experiments with the exception of the last ration experiment, as described below (Table 1, Table 2). Tagging of rainbow trout facilitated the measurement of growth in individual fish. Males and females showed consistent patterns of growth over the experiments. Significant decreases in growth during the effluent exposure were noted to be statistically significant in the 2000 and 2002 experiments, with a similar trend seen in 2001. These were not interpreted as an effluent-associated physiological effect, and it is likely that the reduced water clarity due to effluent colour increased the probability of food pellets reaching the bottom of the tank without being consumed. Ration experiments confirmed that growth differences were most apparent at higher rations and not at lower rations (van den Heuvel et al. 2008).

Liver MFO activity, as measured by the 7-ethoxyresorufin-O-deethylase (EROD) endpoint, was measured in both males and females at the termination of all experiments. EROD is a well established indicator of exposure to a number of organic compounds, and while it is not a measure of toxicity itself, it may be related to other mechanisms of toxicity, depending on the class of compound causing the induction. Females typically have virtually no EROD activity during the prespawning period, so all comparisons are performed using males (van den Heuvel et al. 2004). This effluent consistently caused a relatively low but statistically significant level of induction until 2001, when EROD induction could no longer be detected.

**Trout Egg-Hatching Studies**

Trout egg fertilization and hatching was performed using fertilized gametes from adult trout exposed in mesocosm experiments. Data is presented from 1999 and 2000 experiments only due to experimental failures in other years. The relative impact of maternal exposure (mesocosms experiments) versus direct effluent exposure (conducted in the Rotorua laboratory) was assessed by a comparison of directly exposed eggs and larvae with the eggs and larvae of exposed adult trout that were reared in reference water (Ellis et al. 2005).

In the direct egg exposures, there was no marked effect of water hardening with 15% effluent on the fertility or survival of eggs to 16 d (Ellis et al. 2005). In a subsequent exposure (with hardening in reference water), no significant effects were found on mortality to hatch, time to hatch, length at hatch, mortality to swim-up, mortality to 320 d, or deformity rate at hatch.

<table>
<thead>
<tr>
<th>TABLE 1. Mean (SEM, n) liver somatic indices in rainbow trout exposed to pulp and paper mill effluent over four years of mesocosm experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Males</strong></td>
</tr>
</tbody>
</table>
| Effluent                                                      0.72 (0.02, 25)  
| Reference                                                     0.77 (0.04, 30)  |
|                                                                 |
| **Females**                                                   |
| Effluent                                                      1.19 (0.05, 28)  
| Reference                                                     1.26 (0.06, 25)  |
|                                                                 |
| **TABLE 2. Mean (SEM, n) condition factor in rainbow trout exposed to pulp and paper mill effluent over four years of mesocosm experiments** |
|                                                                 |
| **Males**                                                     |
| Effluent                                                      1.31 (0.01, 40)  
| Reference                                                     1.29 (0.01, 46)  |
|                                                                 |
| **Females**                                                   |
| Effluent                                                      1.33 (0.02, 44)  
| Reference                                                     1.34 (0.01, 43)  |
Exposure of adult trout to 10 and 12% effluent for two months, followed by incubation of the fertilized eggs in reference water, produced no impact on fertility, survival to hatch, survival to swim-up, or length and weight of fry at swim-up (van den Heuvel et al. 2002; Ellis et al. 2005). Exposure of adult trout to 12% treated effluent for eight months prior to egg fertilization also did not result in differing rates of fertility, mortality to hatch, or mortality to swim-up. However, the 8-month maternal exposure did result in swim-up fry that were significantly shorter and weighed less than the reference swim-up fry (Ellis et al. 2005). This difference corresponded to smaller gonads and eggs in the 8-month-exposed female trout described in van den Heuvel and Ellis (2002). Overall, these results demonstrated that this pulp and paper mill effluent is more likely to elicit indirect impacts on progeny size through chronic exposure of adults to effluent during gonadal recrudescence rather than through direct exposure of early life stages to effluent.

Ration/Effluent Interactions Study

The last two mesocosm experiments (2000 to 2003) incorporated the combined effects of energy intake as manipulated by varying food ration with pulp and paper mill effluent exposure over either one or two consecutive reproductive cycles (van den Heuvel et al. 2008). This design was pursued in order to test the hypothesis that elevated energy intake could mask the reproductive effects of Tasman effluent as observed in the previous exposure. These experiments are outlined further here as the long-term data presented above only represent a subset of these data where ration levels were comparable (0.7% of wet body weight).

The ration studies demonstrated that the level of energy intake affected the full range of measured parameters including energy allocation to somatic growth and gonadal development, steroid production, and haematological parameters (van den Heuvel et al. 2008). Increasing ration level expectedly increased growth, condition, and liver and gonad size. Female trout in the higher ration treatments produced more follicles and had larger eggs, investing the same relative proportion of total energy into ovarian development (as per GSI). Sex steroid levels and haematological parameters were also positively influenced by increasing ration level in males and females. By far, the most dramatic impact of reduced ration on reproduction was to substantially reduce the frequency of sexually maturing fish. In trout exposed to the effluent over two reproductive cycles (sacrificed at age four), the frequency of immature females was 1.5, 17.5, and 36% for the high, medium, and low rations, respectively. The effects of effluent exposure were not as marked as those linked to ration level and typically did not manifest until the second ration experiment where trout were exposed through two consecutive reproductive cycles. Those ration experiments served to illustrate that differences in energy intake, which also occur in natural populations, produced effects of far greater magnitude than any observations made in trout due to effluent exposure, but did not appear to change the response of trout to the effluent.

The physiological effects of pulp and paper effluent exposure observed in these experiments were not consistent between the two ration experiments conducted, nor were they consistent with previously observed impacts in similar experiments with this effluent. Effluent exposure over one reproductive cycle did not impact any physiological parameters in trout. However, when effluent exposure was maintained over two reproductive cycles, a new pattern of effluent response emerged (in addition to the dietary effect), including increased condition factor in both sexes, a reduction in red blood cell counts in females only, and increased sex steroids and reproductive investment in males only (van den Heuvel et al. 2008). Effluent was also observed to cause reduced growth in male trout over the two-year exposure. The effects of ration on gonad and liver size were far more obvious and consistent when a longer exposure was employed; thus, it appears to take more than one full year for energy intake changes to be reflected in those particular physiological endpoints.

Laboratory Studies

Laboratory toxicity studies were conducted to assess androgenic and estrogenic effects in fishes exposed to Tasman Mill effluent. While many of these studies were conducted in the laboratory in Rotorua, some of these short-term studies were also conducted at the Tasman mesocosm facility. These studies are also included in this section due to the relatively short-term nature of the experiments.

The androgenicity bioassay used was mosquitofish (*Gambusia affinis*) masculinization. This bioassay takes advantage of the sexual dimorphism in male and female mosquitofish with the male possessing an elongated anal fin which acts as a sexual organ. This organ, called a gonopodium, can be grown in a female exposed to endogenous androgenic compounds (Ellis et al. 2003).

Mosquitofish exposures were initiated in late 1998. Mosquitofish were exposed to Tasman effluent for 21 d with daily static renewal (Ellis et al. 2003). Untreated effluent at a 15% dilution and treated effluent at 15 and 70% dilutions were utilized for those experiments. Female mosquitofish were then judged as showing masculinization or not. A subsequent mosquitofish exposure was conducted in mid-1999. The purpose of this experiment was to examine whether masculinization was associated with hydrophobic compounds in the effluent. As hydrophobic compounds tend to bind to suspended solids, this was tested through glass fibre filtration of the effluent. The hypothesis that plant sterols or their chlorine dioxide oxidation products could be androgenic was tested and no evidence was found implicating those compounds (van den Heuvel et al. 2006a).
In the first mosquitofish experiment, significant masculinization was demonstrated at all effluent concentrations of treated and untreated effluent (Ellis et al. 2003). Treatment appeared to remove some of the androgenic potency of the effluent, though masculinization was still present at environmentally relevant concentrations of 15% effluent. The results of a subsequent experiment revealed that filtration was able to entirely remove the masculinization potential of the effluent.

Tasman Mill effluent was not retested again until 2002 (Bandelj et al. 2006). At this time, a more sensitive endpoint of masculinization was used; the fin ray 4:6 ratio. During masculinization, anal fin ray 4 elongates while fin ray 6 remains unchanged; thus, an increase in the ratio reflects masculinization. Experimental exposures were conducted similar to those used previously, except a 50% vol/vol effluent concentration was used. In the 2002 experiment that used a 50% vol/vol dilution of the Tasman Mill effluent, masculinization was not observed (Bandelj et al. 2006).

During the 2002 exposures, an additional endpoint was examined: in vitro steroid production of mosquitofish ovarian follicles. This endpoint involves the culturing of ovarian follicles for 18 h in media and subsequently measuring the sex steroid hormones produced as a measure of the steroidogenic capacity of the organism. Mosquitofish exposed to Tasman Mill effluent had significantly lower testosterone production than the reference group.

In 2004, it was possible to capture a sample of mosquitofish directly from the Tarawera River from the receiving area below the Tasman Mill effluent outfall. Masculinization of female mosquitofish was compared with that of a Lake Tarawera population, and it was found that there was no evidence of mosquitofish masculinization in the Tarawera River (Bandelj et al. 2006).

During the mosquitofish exposures from 1998 to 2002, a measure the androgenic potency of the effluents was conducted using in vitro receptor binding techniques (Ellis et al. 2003; Bandelj et al. 2006). These techniques involved chemical extraction of the effluent, and concentration of the extract followed by a measure of the ability of the extract to displace radiolabelled steroid from a receptor preparation. In earlier studies a goldfish testes androgen receptor preparation was used, and in later studies a rainbow trout brain androgen receptor preparation was used. These two androgen receptor sources vary in their relative binding of individual steroids. However, the levels measured should be roughly comparable. The total androgen equivalents measured in 1998/1999 was 155 ng/L as testosterone, and in 2002 was 87 ng/L as testosterone (with upstream Tarawera River reference water measuring 17 ng/L). Though this decrease seems insufficient to explain the decline in mosquitofish masculinization, it must be realized that the relative potency of individual compounds in the in vitro bioassay may vary considerably from that in the mosquitofish bioassay.

Trout Estrogenicity Studies

The expression of vtg was measured in a number of short-term and long-term rainbow trout bioassays (Table 3). Out of five trout effluent exposures in which vtg was measured, vtg was only significantly induced on one occasion. In 2002, estrogen receptor agonists were measured in Tasman Mill effluent by methods similar to those described for androgens above. The estradiol equivalents in the Tasman Mill effluent were measured at 35 ng/L compared with 4.8 ng/L as estradiol equivalents in the upstream Tarawera River water (Bandelj et al. 2006). Taken together, these results suggest that estrogen agonists may be present in the Tasman Mill effluent at low levels and only occasionally reach sufficient levels to cause biochemical effects in fishes. It should also be noted that human sewage was removed from the Tasman Mill effluent stream in 2002, and human sewage effluents are known to be significant sources of estrogens.

Field Studies on the Tarawera River

Common Bully Population Surveys

A small-bodied native goboid fish, the common bully (Gobiomorphus cotidianus), was chosen as a monitoring species in the development of wild fish monitoring protocols for New Zealand. Common bully population surveys commenced in January 2003, and surveys from nearly 20 sites across New Zealand indicated that spawning of the common bully generally takes place in early summer. Thus, to obtain prespawning bullies with significant reproductive (gonadal) development, November to January was indicated as the best sampling period. Wild common bully were only sampled downstream of the Tasman Mill effluent outfall at this time.

### Table 3. Summary of vtg measurements in rainbow trout

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Life stage</th>
<th>Vtg</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>320 d (15% effluent)</td>
<td>Egg to juvenile</td>
<td>No vtg induction</td>
<td>Ellis et al. 2004</td>
</tr>
<tr>
<td>28 d (10% effluent)</td>
<td>Juvenile</td>
<td>No vtg induction</td>
<td>Ellis et al. 2004</td>
</tr>
<tr>
<td>21 d (10, 30, 70% effluent)</td>
<td>Juvenile</td>
<td>No vtg induction</td>
<td>Ellis et al. 2004</td>
</tr>
<tr>
<td>93 and 188 d (15% effluent)</td>
<td>Mature males</td>
<td>vtg induction</td>
<td>van den Heuvel and Ellis, 2002</td>
</tr>
<tr>
<td>53 d (10% effluent)</td>
<td>Mature males</td>
<td>No vtg induction</td>
<td>van den Heuvel et al. 2002</td>
</tr>
</tbody>
</table>
The initial survey of common bully revealed that upstream bully had substantially greater (10-fold) ovary size than downstream bully (van den Heuvel et al. 2007). It appeared from these results that either the downstream population was not reproducing at the same time, or else downstream populations had a complete failure of reproductive development. In either case, the remainder of the data such as liver size and condition factor could not be interpreted since these factors change with stage of development in fishes. The exception to this was MFO activity which was induced in downstream common bully. This result precipitated over two years of subsequent investigation to explain the dramatic differences in these populations. A number of hypotheses were sequentially explored to explain these results, including 1) populations differed significantly in age or age/size structure, 2) bully migrated upstream to spawn, and 3) populations had a different annual spawning cycle, and thus represent reproductively isolated subpopulations.

Age-length relationships between the upstream and downstream populations were not significantly different, and tissue analysis for stable isotopes of nitrogen and carbon showed that the upstream and downstream populations were clearly spatially distinct (exposure to pulp mill effluent causes a clearly distinct carbon isotope signature downstream). Bully sampling in July 2003 showed normal gonad development at the downstream sampling site. Thus it was confirmed that upstream and downstream populations had different reproductive timing.

This in turn led to a new series of questions as to why reproductive timing differed since the downstream Tarawera River population was the only one known in New Zealand to exhibit this trait. The two possibilities were that this was a specific adaptation to effluent exposure, or that it was a natural occurrence due to local subpopulations. Evaluation of a population of common bully from the Rangitiki River revealed that they had a similar January reproductive status to the Tarawera population (as sampled in January 2004). Since these populations appeared comparable in reproductive timing, it was concluded that the downstream Tarawera bully had a significantly lower condition factor than the Rangitiki River fish. Subsequent evaluation focused on the possibility that upstream and downstream populations formed two genetically distinct populations.

To facilitate genetic analysis, two additional sites were included (Michel et al. 2008). A statistical simplification (of over 180 polymorphic genetic markers) of the anonymous genetic techniques used (AFLP or amplified fragment length polymorphism) indicated two main genotypes. The downstream Tarawera River and Rangitiki River populations were dominated by one genotype, whereas the Lake Tarawera, upstream Tarawera River, and Kaituna River were all dominated by a second distinct genotype; thus there was a clear genetic differentiation between upstream and downstream in the Tarawera River, Lakes Rotorua and Tarawera were known to be stocked with forage fish of Waikato River origin in the early 1900s. While gene flow in an upstream direction is unlikely due to physical barriers (e.g. waterfalls), gene flow in a downstream direction is feasible.

The results of these combined genotype and phenotype analyses show distinct populations in the downstream Tarawera and Rangitiki Rivers dominated by diadromous bully (Michel et al. 2008). It was speculated that the dominant genotype in the upstream Tarawera River population was owing to both dominant recruitment from the upstream Lake Tarawera population and an absence of diadromous bully recruitment due to inland distance. However, Rangitiki River data from a recent study indicate that diadromous common bully may recruit at least 40 km inland, and the absence of diadromous recruits in the upstream Tarawera could suggest that factors additional to inland distance influence fish migration in the Tarawera River system (Bleckley et al. 2009). Michel et al. 2008 showed significant gene flow occurred down the Lake Rotorua-fed Kaituna River (at one of the closest sites to the sea), whereas gene flow has been limited down the Tarawera River. In this regard the Kaituna and the Tarawera Rivers are perhaps the only analogous sites in the country since both originate from lakes stocked with bully from the Waikato River.

One speculative possibility is that periods of hypoxia in the downstream Tarawera River favour the winter spawning diadromous stock when oxygen would not be reduced, helping larval survival, or additionally, effluent avoidance could influence migration in the Tarawera River (Bleckley et al. 2009). While questions remain, it is apparent that the Rangitiki River was the only known site in New Zealand that could provide an appropriate reference site with regards to fish health studies.

Subsequent comparisons of the health of common bully using the Rangitiki River as the reference location were conducted from January to December 2007 (Bleckley et al. 2009). Bimonthly analyses of gonad growth confirmed that bully in the downstream Tarawera and Rangitiki rivers possessed similar reproductive timing, with spawning occurring in spring while both upstream populations indicated summer spawning. Gonadal recrudescence seems to occur at a similar time, but later spawning populations seem to maintain their gonads longer before maturation.

An in depth analysis was done of bully physiological parameters in July when both downstream Rangitiki and Tarawera river populations were both in prespawning condition. Comparisons of prespawning bully showed that the Tarawera River population had higher (8.6%) GSI than the Rangitiki population (5.4%), though it appeared that the Tarawera bully were slightly advanced in spawning time since the Rangitiki population reached a maximum GSI of 8% in September (Landman et al. 2010). Thus it appears that there was no evidence of reduced reproductive growth in the effluent-impacted downstream Tarawera River population. Ovarian follicle size was histologically examined and found to be the
Same between sites. The ability of the ovarian follicles to produce sex steroid hormones was also measured in July, and the Tarawera bully showed some statistically significant increases in steroidogenic capacity, which is likely consistent with their slightly advanced reproductive timing.

There were also no differences in bulky body condition or liver size during the July 2007 sample period. Haematological parameters were examined and there were no differences in red blood cell parameters. Female bully had significantly higher counts of white blood cells. Hepatic MFO activity was significantly induced in male and female bully below the Tasman Mill effluent outfall, indicating exposure to classes of organic compounds that elicit this response. Neutral metabolites of resin acids such as retene were detected in sediment below the mill outfall at the time of fish collection, and this compound is known to cause EROD induction.

**Chronological Summary**

Summarizing over a decade of fish health research on the Tasman effluent is very challenging due not only to multiple species and testing methods, but also due to the long-term and day-to-day changes in effluent quality that occurs at every pulp mill. With ongoing mill changes, effects observed in the mid-1990s are not necessarily relevant to the current or future situation in the Tara River. Thus, results must be put in context of the chronology of the studies, the long-term effluent trends, and effluent quality at the time of the experiments; this section will attempt to synthesize these data in the context of these factors.

**Effluent Quality**

While bulk chemistry parameters are continually monitored by the mill, there has not been a consistent monitoring program that examined detailed effluent chemistry. The most consistent efforts were weekly samples taken during the mesocosm studies from 1998 to 2003 (van den Heuvel et al. 2004). Other than these, chemistry has been sporadic. An examination of the concentrations of the three main extractive classes (resin acids, neutrals, and sterols) revealed a generally flat or slightly declining trend for resin acids, and a potential decrease in neutrals and sterols over the 1998 to 2003 period (van den Heuvel et al. 2004). This should be interpreted cautiously since the lower concentrations of neutrals and sterols often challenge detection limits. Chemical evaluation performed during the 1998 to 2003 exposures had an almost 10-fold lower detection limit than the standard analysis methods due to the prefiltration of 1 L of effluent. A second important observation was made from the detailed 1998 to 2003 data, and that was the extreme week-to-week variability that occurs in effluent chemistry (van den Heuvel et al. 2004). The implication of this is that any biological assay based on a grab sample may not be representative of the average effluent chemistry. Furthermore, it can be concluded that frequent sampling is required to obtain a true picture of trends in effluent chemistry.

During the 1998 to 2003 mesocosm studies, it was noted that the relative abundance of individual resin acids can provide a “fingerprint” that may be diagnostic of changes in mill operations or effluent treatment. While the absolute concentration of resin acids and other extractives changes dramatically from day to day, the relative composition of the major extractive components is somewhat more constant. It is apparent from the resin acid profiles that there were two major changes over the 10-year period examined (van den Heuvel et al. 2004). The first substantial change in 1998 was the virtual elimination in chlorinated resin acids that occurred when bleaching with molecular chlorine was discontinued. The second change occurred at around the time of full screen room closure in 2000. Prior to this change, the abietane resin acids, abietanic, and 13-abietenoc acid, represented almost 40% of the total resin acids. This changed substantially and permanently in 2000, and abietic acid became the predominant resin acid.

While no biological interpretations can be made regarding the changes in resin acid profiles, biological effects of pulp and paper effluents are generally thought to be mediated by the organic extractives (Hewitt et al. 2008). The studies from 1998 to 2003 separated organic extractives into filterable (glass fibre type C) and non-filterable portions (van den Heuvel et al. 2002) and found that 34 to 97% of resin acids remained on the filter paper (with a mean of 75%) and greater than 85% of neutrals and sterols partitioned onto solids. Similarly, a significant fraction of nutrients have been found to be associated with solids (Slade et al. 2004). Thus, in general, extractives and nutrients will correlate with organic solids, though not with inorganic solids. While volatile suspended solids would be the best predictor, routine total suspended solids (TSS) measurement is conducted by the mill. Given that losses of inorganics in lime are sporadic, the use of TSS would serve to increase the variability in any relationship with extractives. Stormwater can also enter the treatment ponds and could be a source of TSS. To examine the load of pulp and paper organics, biological oxygen demand (BOD) was also examined over the time frame of the studies at hand. Recent unpublished studies (Kovacs et al. In press) have shown BOD to be a good correlate of reductions in fathead minnow egg laying.

Over the decade from 1998 to 2008, daily TSS and BOD measurements showed a statistically significant decrease (Fig. 3, $p < 0.001$). This decrease, representing a magnitude of about 25%, should be roughly proportional to a similar decrease in organic extractives and nutrients. The improvement in TSS concentrations cannot be solely attributed to major in-mill changes, but are likely reflective of better treatment system maintenance over the past decade. The most substantial part of the TSS decrease occurred during the first half of the last 10 years, while BOD reductions were more apparent in the latter half of this period.
Reproductive Impacts of a New Zealand Pulp Mill Effluent

Fig. 3. Trends in A) total suspended solids and B) biochemical oxygen demand at the Tasman Mill, 1998 to 2008.

Chronology of Biological Effects

The studies described herein were primarily based on a research program, not on a monitoring program. While this is advantageous in terms of ensuring the rigor of the work through peer review, it is disadvantageous in that the same endpoints were not generally measured in the same way over the duration of these studies. A chronology was prepared which summarized the main effects of either exposure to Tasman effluent (only at environmentally relevant concentrations), or to the receiving environment in the river (Fig. 4).

From this chronology we could draw a number of conclusions. Firstly, MFO activity, a known biochemical indicator of effluent exposure, no longer occurred in direct Tasman Mill effluent exposures after screen room closure in 2000. However, MFO induction did occur in eel and bully, caged or collected in the field after this time (van den Heuvel et al. 2006b, 2007; Landman et al. 2010). This suggests an effect of either other effluent(s) entering the river, or else an effect of exposure to accumulated solids in the river (which did not occur in laboratory and mesocosms exposures). The resin acid neutral, retene, is a known MFO inducer, and the neutrals appeared to be declining in this effluent but continued to be measured in downstream sediments. While MFO induction has not been clearly mechanistically linked to more relevant biological effects, there has always been a strong association. The elimination of MFO induction does eliminate the possibility of effects occurring through this mechanism but not through other mechanisms.

A number of reproductive effects, namely mosquitofish masculinization and depressed gonad size in female trout, also disappeared after mill screen room closure. Some reproductive effects emerged, including reduced in vitro steroid production in mosquitofish and an apparent stimulation of reproductive function in male trout. In this latter case it is difficult to conclude whether this is a new pattern of response, or just due to the fact that this was a longer exposure. Despite these altered responses, it appears that some factor responsible for alterations in reproduction was removed at the time of screen room closure.

A large part of the variability from experiment to experiment can be explained by the dramatically varying effluent strength (concentration of organics), which is often not measured until experiments are completed. These rapid changes in effluent strength can be observed in the concentrations of extractives presented in the above section. As another example, two successive experiments in the chronic dissolved oxygen studies showed a five-fold difference in effluent strength as measured by organic extractives (Landman et al. 2006). To a large extent, this effluent variability is averaged out by endpoints that develop over the longer term, such as condition factor, growth, and energy allocation for gonads, rather than by endpoints that respond rapidly. Mesocosm studies were particularly powerful in that they average the effects of varying effluent quality and provide an answer that is specific to the effluent being tested at the time it is being tested, as opposed to legacy contaminants or other sources of contaminants that may be present in the river. However, they lack the relevance of the receiving environment itself.

Given the variability inherent with the biological testing of effluents and the strength and dilution of the Tasman Mill effluents, it is not surprising that some sublethal effects should come and go. For example, resin acids are generally considered to be the major factor in acute lethality of softwood effluents. Acute lethality of resin acids as measured by the LC50 (median lethal concentration) generally occurs just above 1 mg/L. Full strength Tasman effluent averages just below 1 mg/L of total resin acids. While sublethal effects of resin acids are reported as low as 20 μg/L (Oikari et al. 1983), an acute to chronic ratio of 10:1 is probably more realistic—meaning a conservative effects threshold of about 100 μg/L for resin acids. With a 5 to 15% dilution of effluent in the river, and significantly varying effluent strength, the concentration of resin acids would hover around the threshold for sublethal effects.

Efforts to measure effects on fish populations in the river itself have been difficult. However, on some occasions there appears to be a pattern of lower condition factor in the river as compared with reference locations. This is not surprising given that habitat available for most species of fishes comprises a 1- to 2-m section at the edges of the river. This would suggest overall lower food availability. This was not the case with recent studies on the common
bully since condition did not differ in comparison with the Rangitiki River populations. This study also showed for the first time that reproductive development of bully in the lower Tarawera River is normal. The downstream Tarawera River is substantially modified and fish capture is generally difficult due to a low density, high flow, moving pumice bed, and the almost complete absence of habitat where biota can seek refuge. Thus, the response in the downstream Tarawera River is probably more influenced by habitat than by the effluent itself.

The overall trend presented in this report is one where an initially good quality effluent continues to show gradual improvement in terms of both chemical and biological endpoints in fishes. Clearly, the studies presented in this report were limited in their scope to physiological effects in fishes. While fishes can be powerful monitoring species, their study is only intended to represent one component of a comprehensive environmental effects monitoring program. The detailed study of the Tasman Mill effluent, while useful, has come at the expense of a detailed ecological study of the receiving environment itself. The counterpoint to this is that we can make a relatively strong conclusion about the quality of the Tasman Mill effluent and its potential effects—a very difficult task to accomplish with in-river studies.

**Conclusions**

Over the time period encompassed by these studies, a considerable body of literature has also been published derived from research in North America and Scandinavia. Currently, new research is also rapidly emerging from South America. The literature is too extensive to review in detail here, and has been previously reviewed on a number of occasions over the past decade and a half (Kovacs et al. 1997, 2005; Munkittrick et al. 1997, 1998, 2003; van den Heuvel 2004; Hewitt et al. 2008; van den Heuvel 2010). This body of knowledge has been further enhanced by the Canadian Environmental Effects Monitoring program for pulp and paper effluent, the only mandatory program of its type in the world. The Environmental Effects Monitoring program has been active since the early 1990s and provides a long-term dataset of monitoring for every pulp and paper mill in Canada (Lowell et al. 2005). Thus, we will conclude only on aspects of the Tasman mill studies that are unique, or add significantly to the international body of literature.

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<table>
<thead>
<tr>
<th>Year</th>
<th>Laboratory Studies</th>
<th>Mesocosm Studies</th>
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*Fig. 4. Chronology of biological effects of Tasman Mill effluent (laboratory and mesocosms) and field monitoring results in fishes.*
One of the unique conclusions to come out of this body of work was that observation that genetic difference can be important in environmental effects monitoring (van den Heuvel et al. 2007; Michel et al. 2008). In this case, genetic difference and the resulting phenotypic reproductive changes prevented an upstream-downstream comparison. However, there was still uncertainty as to whether or not effluent was preventing the mixing of those genotypes. To our knowledge such observations have not previously been observed with regards to the environmental monitoring of the effects of pulp and paper effluents in fishes. However, genotype environment interactions is an emerging area of interest, and relationships between genotype and the ability to survive adverse conditions (Devlin et al. 2004) and potential adaptations to environmental pollution have been documented (Fisher and Oleksiak 2007).

The mesocosm studies conducted as part of the Tasman Mill program also contributed to our limited understanding of the interactions between modifying factors and effluent impacts. The first such modifying factor was the timing of exposure, and earlier mesocosm studies suggested that effects on female gonads were only manifest when exposure was initiated before the onset of vitellogenesis. A second modifying factor examined was energy intake, and there has also been a limited body of research in this area. Previous experiments with dietary manipulation experiments showed that pulp mill effluent exposure increased energetic or metabolic demand in brown trout (Salmo trutta) (Vuorinen and Vuorinen 1985) and more recently in rainbow trout (Mattsson et al. 2001). While there were subtle and inconsistent indications of this in the Tasman Mill studies, the larger question was whether varying levels of energy intake (likely to occur in wild populations) could influence how trout responded to the effluent. From this perspective, energy intake did not appear to substantially influence the response of fish to the effluent treatments.

One major conclusion can be drawn from this body of knowledge: subtle effects on fish physiology, including reproductive changes, continue to be observed but have substantially diminished as effluent quality improves. This conclusion was accomplished through focused mesocosm and laboratory studies, and the lack of effects was confirmed using a field model. However, the field studies conducted indicate the full range of difficulties inherent in field studies, including the appropriate choice of reference locations, separating present-day effluent effects from historic contamination in sediment, sampling in challenging aquatic environments, and separating the effects of multiple sources of contaminants. Many of those challenges were overcome by adopting a small-bodied fish model, an approach that also improved the success of the early cycles of the Canadian Environmental Effects Monitoring program.

Thus, the trends in effects internationally, and how we measure them, have paralleled our experience with the Tasman Mill effluent. Research on the identity of bioactive compounds in effluent is continuing, but it is apparent from both the Tasman Mill and international studies that this knowledge is not required to eliminate problems. Limiting wood extractives from reaching the environment through either elimination of their discharge to sewer, or through consistent treatment is sufficient to eliminate biological effects in fishes.

References


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Recent Progress in Understanding the Causes of Endocrine Disruption Related to Pulp and Paper Mill Effluents

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Reproductive impairment in fishes exposed to pulp and paper effluent has been observed in the wild for three decades. Despite intense research, identification of causative agents, if indeed the changes are strictly chemically mediated, has yet to be achieved. This review examines the body of research developed over the last six years that was directed at understanding the mechanisms of reproductive dysfunction in fishes associated with pulp mill effluent exposures. Research has continued to show diminishing effects of effluent exposure on fish reproductive physiology. Observations of exposure to androgens and estrogens continue to be observed and new evidence suggests that antiestrogens may be present. The presence of androgenic steroids remains a consistent observation, and those androgens appear to be native to wood. Recent studies have also suggested a number of alternate mechanisms beyond androgens or estrogens. One such possibility is that neuroactive substances are interfering with endocrine balance critical to reproduction. A second possibility is that some reproductive effects in the field may be due to nutritional factors, and thus reproductive impacts are caused by indirect effects of pulp mill effluent exposure. Ongoing mechanistic studies, particularly with paired field-lab components, are required to make further progress.

Key words: fish, reproduction, pulp and paper, endocrine disruption, androgen, estrogen

Introduction

The series of three-yearly international conferences on the environmental fate and effects of pulp and paper effluents was initiated in response to rising concern about the potential environmental impacts of the industry. The emerging literature of the late 1980s that documented the impacts of pulp and paper mill effluents on fishes contributed significantly to the creation of these meetings. Though the nature of effluent, endpoints, and issues have changed significantly since the first conference, fish health, and in particular reproductive fitness of fishes, has consistently been a strong focus of these conferences.

At the first meeting held in Saltsjobaden, Sweden in 1991 (Södergren 1992), there was a strong focus on the biochemical and physiological responses in fishes collected from the receiving environments of a limited number of bleached kraft mills. The industry was well on its way to addressing the release of organochlorines, including polychlorinated dibenzo-p-dioxins and dibenzofurans. Such was the success of conversion to chlorine dioxide that in Vancouver, Canada (Servos et al. 1996), the discussion had shifted to the relative impacts of elemental chlorine free bleaching (ECF) versus totally chlorine free bleaching (TCF). At this time, interest in the effects of nonchlorinated compounds on fish health was still limited. At the Rotorua, New Zealand meeting in 1997 (Stuthridge et al. 2003), the emphasis of research had shifted again and the attention was focused on defining and measuring meaningful effects, with significant emphasis on both field and laboratory fish studies. At all of the last three conferences—in Helsinki, Finland in 2000 (Ruoppa et al. 2000), Seattle, U.S.A. in 2003 (Borton et al. 2004), and Vitoria, Brazil in 2006—the studies on aquatic impacts in fishes have shown a strong continuing focus on reproductive-endocrine impairment.

The study of the reproductive effects of pulp and paper mill effluent on fishes is certainly among the largest single body of research on endocrine disrupting substances in the environment. At this point in time, we know that fishes experience reductions in their reproductive potential globally. There is also evidence to suggest that such effects are gradually diminishing as the industry continues to improve effluent quality. There is no single compound or group of compounds that appear to be unambiguously related to the reproductive effects observed around the world. However, the ability of diverse effluents to cause reproductive dysfunction and the persistence of the issue after the reduction of organochlorines, combined with research on the effect of process and treatment changes (Hewitt et al. 2008), have given a clear indication that the nature of causal agents is wood related.

Despite the large body of research, we do not know if there is one common mechanism or a single group of causative agents, or merely a diverse array of reproductive responses to a complex mixture of different compounds. The purpose of this review is to evaluate evidence from the past six years on the reproductive impacts of pulp and paper mill effluents on fishes. This is not a comprehensive review on all of the literature over that period, but an assessment of select research where effects can be categorized according to potential causal
agents or mechanisms of action. The review is organized into the broad categories of estrogens, androgens, and alternate potential mechanisms.

**Estrogens**

Unlike sewage effluents, pulp and paper effluents are not expected to contain substantive quantities of estrogenic pharmaceuticals, though there is longstanding evidence that effluents can contain estrogen agonists (Mellanen et al. 1999; Tremblay and Van Der Kraak 1999). However, the observations of estrogenic effects have not been consistent, not even within a single effluent (van den Heuvel et al. 2010a).

While evidence of estrogens and androgens is described in detail in two separate sections herein, data for both estrogens and androgens are summarized in Table 1 since many studies measured multiple endpoints. Most evidence of estrogen exposure has been derived from the induction of the egg yolk precursor protein vitellogenin (vtg) since the expression of this protein is under the direct control of estrogens. The most recent evidence of estrogens in pulp and paper effluent has arisen out of studies of Chilean mills (Orrego et al. 2003, 2006, 2009). These studies examined the effect of four mills situated on the Biobio River, Chile. All mills used a mixture of pine and eucalypt, were ECF bleached kraft, but not all had secondary treatment. Initial studies examined the laboratory exposure of rainbow trout (Oncorhynchus mykiss) to sediment collected upstream and at two points downstream of the four mills (Orrego et al. 2005). Significant vtg induction was measured in trout exposed to both the near downstream and the far downstream sediment. A second study on the same sites used in situ caging and laboratory exposure of trout to effluent and found a similar vtg induction pattern (Orrego et al. 2006). A final study examined organic extracts of two of the pulp mills on the Biobio River. The effluent extract from both mills tested, from a primary- and a secondary-treated effluent, showed significant vtg induction that peaked at four to seven days post injection of the extract. This series of studies demonstrated that estrogens were consistently present in both sediment and effluent associated with the Chilean pulp mills examined. A Scandinavian study examined vtg induction in effluent-exposed zebrafish (Danio rerio) and found vtg induction at 50% vol/vol dilution of effluent (Orn et al. 2006). The mill was a TCF kraft mill with secondary treatment, but no indication was given as to wood furnish.

<table>
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<tr>
<th>Study</th>
<th>Mill and location</th>
<th>Wood</th>
<th>Process</th>
<th>Treatment</th>
<th>In vitro (as ng/g, E2 or T)</th>
<th>In vivo</th>
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<td>+ vs</td>
<td>ND</td>
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<td>NP</td>
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<td></td>
<td>4. Chile</td>
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<td>NP</td>
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<td>AS</td>
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</table>

*The specific nature of the tests used is described in the text. Where possible, measured concentrations of in vitro determinations are given. For in vivo tests, the lowest estrogens concentration at which significant effects were observed is listed.

1 Where quantification was not possible, positive (+ vs) was indicated where statistically significant differences were observed, or negative (− vs) where there was no significant difference.

2 Results from the first two mill are due to direct injections with effluent extracts, data from the latter two mills are cumulative effects of sediment downstream of all 4 mills, and in situ caging downstream of all mills, so results cannot be directly attributed to any specific mill.
A study conducted in New Zealand examined the potential of components in extracts of three final effluents (one from a New Zealand mill and two from Canadian mills) to bind rainbow trout estrogen receptors (Bandelj et al. 2006). One of the mills was an integrated ECF kraft/TMP (thermomechanical pulping) mill, had secondary treatment, and pulped a mixture of pine and eucalypt. The other two mills were an ECF kraft/TMP mill and a TMP mill, both located in Canada. The Canadian mills largely used a mixture of softwood and both had secondary treatment. The New Zealand mill and the Canadian kraft/TMP mill both showed elevated levels of estrogen receptor binding as compared with upstream water extracts. Total estrogens were measured to be 25 to 35 ng/L as estradiol equivalent concentration. However, the Canadian TMP mill did not show significantly elevated levels of estrogen receptor binding. While receptor binding appeared to be present in two of the effluents, receptor binding does not allow the differentiation of agonists and antagonists. The New Zealand pulp mill effluent had previously been shown to induce vtg in rainbow trout, but only in one of five exposure experiments (van den Heuvel and Ellis 2002; van den Heuvel et al. 2010a). A follow-up study to the above mentioned study examined estrogen receptor binding in a different secondary-treated, softwood, Canadian bleached kraft mill effluent, and estradiol equivalent concentrations ranged from 53 to 93 ng/L as estradiol (Wartman et al. 2009). This effluent was not able to elicit an estrogenic response in vivo even at 100% vol/vol effluent as measured by hepatic vtg mRNA expression in the threespine stickleback (*Gasterosteus aculeatus*).

Only one study has examined the potential of effluent extracts to bind estrogen receptors, and/or act as estrogen antagonists (Marlatt et al. 2006). This study examined the organic extracts of eight different pulp mills, all using a mixture of pine, fir, and spruce and employing secondary treatment. Both rainbow trout estrogen receptor binding and rainbow trout primary hepatocyte vtg bioassays were used to measure estrogen agonists. Through co-exposure with estradiol, the primary cell culture bioassay was also employed to measure estrogen antagonists. Receptor binding showed concentrations ranging from 200 ng/L to over 1,000 ng/L as estrogen equivalent concentration in all eight effluents. However, the rainbow trout hepatocyte vtg bioassay showed that none of those effluents was capable of inducing vtg in vitro. Three of the effluents tested were observed to have antiestrogenic activity. There was no consistent pattern of response based on the pulping process used, and on this basis it was speculated that compounds involved are derived from wood. A very recent study has found resin acids and pulp mill effluent extracts to be antiestrogenic in a yeast-based human estrogen receptor construct (Terasaki et al. 2009). Resin acids and extracts showed no affinity for the estrogen receptor, indicating that in this case antiestrogenicity was independent of receptor binding.

**Androgens**

The androgenic effect of pulp and paper effluent on mosquitofish (*Gambusia affinis*) represents one of the earliest documented cases of endocrine disruption in fishes (Howell et al. 1980). Male mosquitofish possess a copulatory organ that is an extension of the anal fin called a gonopodium. When females are exposed to exogenous androgens, they can irreversibly develop a gonopodium, becoming outwardly masculinized. While this observation was ignored for many years, recent studies confirm that pulp and paper effluent can cause androgenic effects in vivo at mills around the world (Larsson et al. 2000; Ellis et al. 2003). Research at one of the original sites where mosquitofish masculinization was first observed indicates that this effect has diminished significantly in severity (Orlando et al. 2007). Mosquitofish now only show very subtle signs of masculinization in the Fenholloway River, U.S.A. Unfortunately, no conclusions can be drawn regarding the recovery at this site as wood furnish, pulping process, treatment, and any ongoing improvements at the mill have not been reported in the literature.

A study using a Canadian bleached kraft effluent (mixed softwood) with secondary treatment measured exposure to androgens using in vitro receptor binding. Parallel to this, in vivo exposure to androgens was measured using transcripts of an androgen-responsive gene, spiggin, in the posterior kidney of female threespine stickleback using qPCR (quantitative polymerase chain reaction) (Wartman et al. 2009). After a 21-d laboratory exposure, a significant induction in spiggin mRNA expression was observed at 10 and 100% vol/vol effluent. This effluent did not elicit changes to in vitro sex steroid hormone production. Rainbow trout brain androgen receptor binding was examined in extracts derived using either liquid-liquid extraction with dichloromethane or C18 solid phase extraction, both with and without florisil clean-up. The concentrations of receptor binding substances were not greatly influenced by extraction techniques and ranged from 190 to 283 ng/L testosterone equivalent concentration.

A number of in vitro studies have recently been published confirming the presence of androgens or antiandrogens at other pulp and paper mills around the world. Chatterjee et al. (2007) measured androgenic substances in five Indian effluents using a yeast-based human androgen receptor bioassay, but no effluent or mill information was presented in support of these data, and these data were not quantified as an androgen equivalent concentration. A yeast-based reporter gene bioassay was also used by Svenson and Allard (2004) to measure androgens in effluent extracts from six mills. In this case only one of the six effluents was found to be androgenic, but the levels were too low to quantify. However, androgenic substances were found in the bile of fishes caged in those effluents that showed negative effects in the yeast androgen screen. The androgenic
compounds appeared to be recalcitrant with regards to secondary treatment. Örn et al. (2006) measured 5.6 ng/L as dihydrotestosterone in a Swedish TCF kraft effluent in a yeast-based androgen bioassay. Fish androgen receptor binding assays have also been used in a number of studies to measure receptor binding substances associated with effluents. A study of an ECF kraft mill using both hardwood and softwood furnish showed elevated goldfish testes androgen receptor binding substances in effluent extracts (Hewitt et al. 2005). In this case, secondary treatment removed greater than 90% of the binding activity. Androgen receptor binding substances were also observed to accumulate in the tissue of white sucker captured in the receiving environment. Bandelj et al. (2006) measured rainbow trout androgen receptor binding in effluent extracts from three mills described in the previous section on estrogens. Concentrations ranged from 50 to 100 ng/L as testosterone for all three mills. Only one of the mills was tested for its ability to cause mosquitofish masculinization, and no masculinization was found to occur at 50% vol/vol effluent or in field-captured samples (approximately 10% vol/vol).

A number of theories have been presented as to the identity of androgenic substances in pulp mill effluents. The steroids androstenedione and androstadienedione have previously been implicated as androgenic compounds in pulp and paper effluent (Jenkins et al. 2001), though there was some dispute regarding this observation (Durhan et al. 2002). The authors postulated that those steroids were microbial breakdown products of plant sterols such as sitosterol, though such a biotransformation has not been experimentally demonstrated in pulp and paper treatment systems or in the receiving environment. More often than not, androgenic activity has been found in primary effluents and is reduced by secondary treatment (Ellis et al. 2003; Hewitt et al. 2005). Other studies on a number of pulp and paper mill effluents have failed to find androstenedione and androstadienedione (Ellis et al. 2003) and other steroids using GC-MS SIM (gas chromatography-mass spectrometry in Selected Ion Monitoring mode) analysis that screened for 26 steroids (Bandelj et al. 2006) and 39 steroids (Wartman et al. 2009). The two androgens implicated also require higher concentrations to masculinize mosquitofish than were found in receiving waters (Bandelj et al. 2006).

Recent evidence has led some investigators to modify their previously proposed hypothesis that the breakdown of plant sterols to androstan steroidal steroids is the source of androgens in pulp and paper effluent. Since the initial discovery of androstenedione and androstadienedione, Carson et al. (2008) measured progesterone in loblolly pine (Pinus taeda L.) and suggested that progesterone is the precursor of steroids subsequently produced by microorganisms. However, there is a large body of research that has documented androgenic compounds in the tissue of pine and other plant species. One study identified dehydroepiandrosterone, dihydroxyetiocholanolone, epistosterone, 5-androstenediol, and pregnenolone as androgenic impurities in a purified commercially available source of sitosterol (van den Heuvel et al. 2006). It was only after the publication of this work that the authors realized that the sitosterol preparation was derived from wood (Steraloids, personal communication), likely softwood tall oil, a common source of sitosterol. Two of those same compounds have been reported as androgenic impurities in plant sterol-based health products derived from pine (K. Pegel, South Africa, personal communication). Larsson et al. (2006) examined androgen receptor binding in effluent extracts from a secondary treated TCF kraft pulp mill using largely spruce and pine, and found receptor binding substances were removed, not created by treatment; though the receptor binding steroids remain unidentified, progesterone was present in primary effluent.

A review of the wood chemistry literature shows that androgens were known to be present in wood, particularly pine species, for decades. Two examples include testosterone, epitestosterone, and androstenedione, which were measured in scotch pine (Šaden-Krehula et al. 1971), and 11-ketoandrosterone, 11-ketoetiocholanolone, 11-hydroxyetiocholanolone, etiocholanolone, androsterone, dehydroepiandrosterone, and adrostenedione, which were all found in the pollen of Pinus nigra (Šaden-Krehula and Kolbah 1983). While androgens are traditionally viewed as vertebrate steroids, it is now clear that these compounds are produced in and play physiological roles in plants. Several of those 11-ketosteroids are corticosteroids and thus could have the potential to influence cortisol function. Estradiol has also been observed in Pinus tabulaeformis pollen (Zhang et al. 1991), and Simons and Grianwich (1989) found estrogens and androgens in 128 species from 50 plant families, including Pinus banksiana, Pinus sylvestris, and Pinus glauca that had among the highest levels of androstenone of any species tested, though an aspen species was also equally high. Thus, there is currently a substantive body of evidence to suggest that androgens can originate in wood, and that secondary treatment can partially or totally remove those compounds; there is no direct evidence that androgens are being created in treatment systems or the environment by microbial action.

**Alternate Mechanisms**

There has been an appreciable amount of research effort on measuring androgens or estrogens in relation to reproductive effects of fishes exposed to pulp and paper mill effluent, but limited effort on other potential causative agents and/or mechanisms. A New Zealand study found significantly reduced carotenoids in the ovarian follicles associated with reduced in vitro sex steroid hormone production in a native goboid fish—the common bully (Gobiomorphus cotidianus) (Landman et al. 2008)—exposed in the wild to a bleached kraft mill effluent. While not previously quantified, pale

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*van den Heuvel*
ovary colouration had for many years been observed in white sucker (*Catostomus commersoni*) captured in the receiving area of a bleached kraft mill that discharged into Jackfish Bay, Lake Superior, Canada (KR Munkittrick and ME McMaster, unpublished data). Studies at another Canadian bleached kraft mill demonstrated profound retinoic acid depletion in white sucker livers (Alsop et al. 2003). Retinoic acid is responsible for most of the activity of vitamin A, particularly in growth and development, so such a nutritional deficiency may have significant reproductive consequences. While the exact function of carotenoids in the eggs of fishes is uncertain and potentially multifaceted (Goodwin 1986), carotenoids may be linked to the retinoic acid pathway as precursors. Carotenoids may also be involved in protection against reactive oxygen species. This could be particularly relevant since evidence of oxidative stress has been documented in white sucker exposed at the Jackfish Bay pulp and paper mill (Oakes et al. 2003). Carotenoids are known to be produced only by plants and it has been speculated that light attenuation in the receiving area of pulp mill effluents favours heterotrophs over autotrophs, thus leading to a depletion in carotenoids in the food web. While it is not known if carotenoid or retinoid depletion could lead to whole organism effects (such as reduced gonad size) in the field, it is noteworthy that such an indirect nutritional effect of effluent could not be easily detected by any laboratory bioassay. Altered ration has been observed to affect steroid hormone production in mesocosm exposures with captive rainbow trout, supporting a potential nutritional mechanism for reduced gonad growth (van den Heuvel et al. 2008).

A recent study has demonstrated the potential of pulp mill effluent extracts to bind goldfish (*Carassius auratus*) neuroendocrine receptors and to inhibit key neuroendocrine enzymes (Basu et al. 2009). Increased or decreased binding was observed in receptors involved in gamma-aminobutyric acid (GABA), dopamine, glutamate, and acetylcholine-dependent neurotransmission. Decreases in the neuroendocrine enzyme activity of monoamineoxidase, GABA-transaminase, and acetylcholinesterase were observed. These neurotransmitter pathways are critical in reproduction and directly up or down regulate the release of luteinizing hormone from the pituitary. This potential mechanism could be involved in the suppression of egg laying, which is seen to occur very rapidly in some laboratory bioassays, but this result needs to be validated at the in vivo level. Effect at the brain and/or pituitary level as measured by limited responsiveness to gonadotropin releasing hormone was indicated in one of the early studies at Jackfish Bay (Van Der Kraak et al. 1992), consistent with the proposed mechanism in the aforementioned study.

Several studies have examined the role of other wood-related compounds as potential endocrine disrupting substances through their ability to decrease steroid hormone synthesis. In one study, mummichog (*Fundulus heteroclitus*) were exposed to black liquor condensates (Belknap et al. 2006). The ability to reduce gonadal testosterone production in vitro was used as the endpoint to track the biological activity of chemical fractions of the black liquor condensates from two bleached kraft mills. A number of potential endocrine disrupting substances were identified from bioactive fractions, including four terpenoids, a 3,5-dimethoxy stilbene derivative, caparratriene, and two C20 acyclic diterpenoid alcohols that were more predominant in softwood than in hardwood. A fifth compound was identified as sulphur (S8) and was found equally in hardwood and softwood. Another study examined the wood derived compounds betulinol and dehydroabietic acid on reproduction in zebrafish (Christianson-Heiska et al. 2008). Environmentally relevant concentrations of those compounds had no effect on reproduction measured as egg laying, reproductive development, or steroid hormone levels in zebrafish over two generations.

**Conclusions and Future Directions**

The past six years have seen a substantial body of research published, giving us clues as to causative agents of reproductive impairment in fishes. It is clear from this research that the manifestation of reproductive effects in fishes may be more complex than previously thought with multiple causes and mechanisms. Different processes, treatments, wood furnish, bioassay test species, and indirect effects in the receiving environment, coupled with the inherently high day-to-day variability in effluent strength and quality make establishing causes and mechanisms akin to the search for the proverbial needle in a haystack. The scientific reporting of biological results has exacerbated the difficulty in interpreting patterns from the literature because wood furnish, mill process, treatment, and effluent chemical characteristics are under-reported, or not documented at all. It is critical as we move forward on this issue that scientific papers not be accepted for publication without a basic set of mill descriptors and effluent background and chemistry.

It has become clear that estrogens, androgens, and other potential endocrine disrupting substances are native to the wood being pulped. For example, it appears that trees, both hardwood and softwood, have some of the highest measured concentrations of steroids among plants. This has several implications, the first being that the effects observed could be specific to the tree species being pulped. For example, with the emergence of a large South American pulp and paper industry, the pulping of eucalypts has increased greatly. Some aspects of the wood chemistry of eucalypts are unique and we cannot assume that such an effluent will have similar effects as a North American softwood effluent. Though patterns between species pulped and effects are not abundantly apparent from the literature, tree species has been the most infrequently reported variable. Mills often use mixtures of wood that vary considerably from day-to-day, and such information is not recorded or is difficult to obtain.
A second implication is that for kraft pulping, controlling liquor loss is a key to preventing these compounds from entering the environment because the bulk of the low molecular weight material is removed in pulping. At a New Zealand pulp mill with secondary treatment, biological effects were eliminated after pulp mill screen room closure, eliminating the loss of weak pulping liquor to sewer (van den Heuvel et al. 2010a). Elimination of wood extractives entering sewer is difficult in the case of TMP effluent. However, there is also abundant evidence that estrogens and androgens and their effects can be removed by efficient treatment.

While the presence of a number of endocrine disrupting compounds in effluent is becoming clear, with some exceptions, the involvement of these compounds in organism- or population-level effects in the field or the lab is unclear. The exceptions are cases, such as the masculinization of mosquito fish or the male-skewed sex ratios in eelpout (Zoarces viviparous), where there is an obvious mechanistic link between the compounds and the effects. In the case of estrogens, while there is chemical and biochemical evidence of exposure, there has yet to be found any indication of whole organism reproductive effects as with the case of municipal sewage (Sumpter and Johnson 2005). In fact, the reduction of gonad size in fishes would more plausibly be caused by antiestrogens, thus the recent characterizations of antiestrogens in pulp and paper effluent may be significant.

One of the challenges facing scientists is linking in vitro bioassays and in vivo biochemical indicators of exposure with whole organism reproductive endpoints in laboratory and wild fishes. This observation is not only true in the study of pulp mill effects, but is globally applicable in environmental toxicology. In vitro endpoints are generally more sensitive than in vivo endpoints and it has been strongly recommended that a combination of these be used in a tiered approach (Kunz et al. 2006). For estrogens, different bioassays vary markedly in sensitivity even within in vitro or in vivo categories (Dobbins et al. 2008). Experiments with the receptor binding of pulp mill effluent extracts and in vivo testing of mosquitofish masculinization have shown discordance between in vitro and in vivo results (Ellis et al. 2003). Interactions between estrogens and androgens may be different in vivo than in vitro as well, largely due to metabolism in vivo but also due to the complexity of multissae hormonal feedback. One such example is the ability of the androgen methyltestosterone to induce vtg in vivo (Hogan et al. 2008). Vtg is generally thought by most to be an indicator of exposure to estrogens, but the aromatization of androgens can occur in vivo, giving misleading indications of exposure to estrogens and further credence to the call for the paired use of in vitro and in vivo endpoints. Collectively, these findings show that caution must be used in the interpretation of results of studies such as are presented in this review.

A second challenge is the uncertainty of whether laboratory bioassays can predict wild fish effects given the substantial number of emergent properties of ecosystems (Kerr 1976). These are challenging questions that require integrated teams of researchers operating in a coordinated fashion in cooperation with industry. As an example in wild fishes, one of the patterns of response observed has been reduced gonad size associated with greater condition and liver size. This has been observed as a prevalent pattern in data generated as part of Canada’s federally mandated Environmental Effects Monitoring Program (EEM) (Lowell et al. 2005). This response has been termed metabolic disruption and is the key response that researchers are seeking to address and eliminate as part of an Investigation of Cause Project (IOC) associated with the EEM program (Parrott et al. 2010; van den Heuvel et al. 2010b). Currently it is still unknown if there is a laboratory bioassay that is capable of predicting reduced gonad size in wild fish populations.

The point may also be made that given our current level of knowledge, we know that a reduction of losses within the mill (including from spills) and efficient and stable effluent treatment is capable of greatly reducing or eliminating reproductive effects in fishes. These two factors are probably the single most important reasons for the diminishing incidence of reproductive effects observed in the field over the preceding decades (Hewitt et al. 2008). While at this point it is not essential that we know precisely what compounds and mechanisms are involved in reproductive dysfunction to solve this issue in pulp and paper, in the long-term this information will be widely applicable. While the timeline may be disputed, the reduction in fossil fuel use for energy and materials will ultimately force us to rely more on plants (especially trees) for those fuels and materials. The development of biorefinery science and technology is evolving at a staggering pace without consideration for environmental effects. Every plant material processing facility will have an effluent, and each of those effluents will have the potential of containing some of the rich diversity of bioactive natural products present in plants, just as we have seen with the pulp and paper industry. Thus, an understanding of causes and mechanisms of reproductive dysfunction in fishes related to pulp and paper effluents will be invaluable in preventing future environmental problems.

References


Masculinization of Eastern Mosquitofish (Gambusia) and Exposure to Pulp and Paper Discharge: Diminished Responses Following Mill Process Modifications

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The abnormal induction of anal fin elongation (masculinization) in female Gambusia was first reported in the 1980s for Florida streams receiving pulp and paper effluents. Although these early reports indicated masculinization responses that were similar to the complete development of a gonopodium (male secondary sex structure), additional evaluations throughout the 1990s demonstrated significant reductions in this response. These historic data suggested that mill process modifications may have been responsible for the reduced masculinization responses. The objective of the current study was to utilize Georgia Pacific’s Palatka Mill to monitor a series of mill process modifications and their effects on masculinization responses in Mosquitofish (Gambusia). Gambusia were collected from upstream, discharge, and downstream sites in the Palatka Mill receiving stream (Rice Creek) and masculinization was evaluated. Collections were conducted annually during 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2008, and 2009, before and after a series of modifications which included: conversion to elemental chlorine free bleaching, conversion of secondary treatment to aerobic degradation, reduction of in-mill black liquor losses, addition of condensate stripping, reduction in water usage from 136 to 83 million litres per day (36 to 22 million gallons per day), installation of new brown stock washers, and oxygen delignification. Masculinization responses were evaluated using the anal fin index (ratio or anal fin rays 4 and 6). Reductions in this masculinization response were observed gradually across 1999 through 2009, with an absence of response in female Gambusia during 2004, 2006, and 2008 to 2009. These data indicate that pulp and paper mill upgrades and process modifications have resulted in the elimination of this biological response in Gambusia.

Key words: masculinization, diminished responses, Gambusia, pulp and paper, fish

Introduction

Mosquitofish are a live-bearing fish species in the family Poeciliidae. Adults are sexually dimorphic; the male is smaller with an elongated anal fin forming a gonopodium or intromittent organ used to attract and inseminate females (Fig. 1). Development of this organ is androgen dependent as the male matures (Ogino et al. 2004). Gonopodial induction or anal fin elongation in adult females has been demonstrated in the laboratory in response to exposure to various androgens directly (Turner 1941a, 1941b, 1942; Angus et al. 2001; Stanko and Angus 2007), and indirectly from bacterially-degraded phytosterols (Denton et al. 1985; Howell and Denton 1989). The induction of anal fin elongation or development of a gonopodial-like structure in female Gambusia has been termed “masculinization.”

Masculinization of wild mosquitofish was originally documented in Elevenmile Creek and the Fenholloway River, Florida, U.S.A. (Howell et al. 1980; Bortone and Drysdale 1981). Females living downstream of pulp and paper discharge in both streams were observed with elongated anal fins, often times resembling a gonopodia of similar length and terminal differentiation to fully mature males. Precocious maturation of males was also reported. Effluent-exposed males began developing gonopodia earlier (at smaller body lengths) than unexposed males. However, these differences were no longer apparent at late-stage gonopodial growth (Drysdale and Bortone 1989). Following major process changes by the mill at Elevenmile Creek, including conversion to elemental chlorine free bleaching and oxygen delignification, the authors concluded that process changes did not influence masculinization effects (Cody and Bortone 1997).

More recently, the masculinization response in Gambusia has been detected in Rice Creek, Florida, U.S.A. (Bortone and Cody 1999). Rice Creek is a tributary of the St. Johns River and the receiving stream for effluent from a Georgia Pacific bleached kraft paper mill at Palatka, Florida. As of the late 1990s, there were two bleached lines (40% product) and one unbleached line (60% product). Bleached lines produced paper towels and bath tissue, while the unbleached line produced kraft bag and linerboard. Furnish for the mill was cycled approximately 50:50 between hardwoods and softwoods. Secondary effluent treatment lagoons/ponds consisted of anaerobic followed by aerobic degradation with approximately a 40-day retention time. Following treatment, effluent was discharged approximately 6 km upstream of the St. Johns River into Rice Creek.

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Fig. 1. Anal fin morphology for adult Gambusia. Females are generally larger in body weight and length than male fish. Male fish have an elongated anal fin, representing an elongation of fin rays 3, 4, and 5, to form a copulatory structure known as a gonopodium. Females normally have no or minimal elongation of these fin rays. An anal fin index, or ratio of length for fin Rays 4 and 6, compares the primary elongated fin ray with a fin ray which does not elongate and provides an index of anal fin elongation, or masculinization.

In 2001, the mill began a series of long-term mill improvements and/or major process upgrades and modifications. Pre-2001, mill processes were pre-Cluster Rule compliant with elemental chlorine bleaching, a mix of anaerobic and aerobic secondary treatment, and water use of 98,500 to 79,500 litres per tonne (26,000 to 21,000 gallons/ton). Improvements since 2001 included: Cluster Rule compliance with conversion from elemental chlorine bleaching to 100% ClO₂ bleaching (2001), improved black liquor recovery and condensate stripping (2001, 2004, 2005, and 2008), secondary treatment pond conversion to 100% aerobic (2001), reduction in water use from 79,500 to 64,500 litres per tonne (21,000 to 17,000 gallons/ton) (2001 to 2003), additional decreased black liquor loss and improved chemical recovery (2004 through 2007), a further reduction in water use from 64,500 to 57,000 litres/tonne (17,000 to 15,000 gallons/ton) (2004 to 2005), replacement of brown stock washers (2007), the addition of two-stage OD (oxygen delignification) (2008), and a final reduction in water use from 57,000 to 49,000 litres per tonne (15,000 to 13,000 gallons/ton) (2006 to 2008).

The objective of the current study was to utilize Georgia Pacific’s Palatka Mill to monitor this series of mill process modifications and their effects on masculinization responses in Gambusia. Gambusia were collected from upstream, discharge, and downstream sites in the Palatka receiving stream, Rice Creek, and masculinization was evaluated annually during 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2008, and 2009.

**Materials and Methods**

Eastern mosquitofish, *Gambusia holbrooki*, were collected during reproductively active months (March through June) during 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2008, and 2009. *Gambusia* were not collected during 2007 due to schedule and budget restrictions. Water quality parameters (dissolved oxygen, temperature, pH, conductivity, and salinity) were measured before fish collection at each site. Adult males and females (50 to 100 each) were captured along shallow vegetated banks using dip nets at an upstream site at the discharge site and at a downstream site in Rice Creek (Fig. 2). Fish were transported to the laboratory in oxygenated bait buckets then euthanized with a terminal dose of buffered tricaine methanesulfonate (Tricaine-S, Western Chemical Inc., Ferndale, Wash., U.S.A.). Body weight (±0.001 g) and standard length (±0.01 mm) were measured using a digital scale and a pair of digital callipers.

Once euthanized, fish were examined under a dissecting microscope to determine gender using the urogenital papilla. Females have a gender-specific urogenital papilla protruding from the urogenital sinus.
This papilla is used by males to anchor the gonopodium during copulation (Meffe and Snelson 1989). This technique of gender identification was validated against macroscopic and histological evaluation of gonads, as well as among personnel (Noggle 2005). It provided a way of distinguishing sex separate from the endpoints of interest.

Linear distance from base to tip of rays 4 and 6 of the anal fin (±0.1 mm) were measured for all fish using a dissecting microscope and an ocular micrometer and/or digital photographs of anal fins and computer assisted measurements using a computer software program (SigmaScan Pro 5.0, SPSS, Inc.) to trace along the lengths of rays 4 and 6 (±0.01 mm). The ratio of anal fin rays 4 to 6 was determined to be the most sensitive endpoint of anal fin elongation and independent of body size, and measurement techniques did not differ (Noggle 2005). Therefore, the 4:6 anal fin ray ratio was utilized as an index of anal fin elongation and masculinization. Photos of normal male and female *Gambusia* as well as a representative masculinized female are summarized in Fig. 3.

In addition to indices of anal fin elongation (masculinization), results were also calculated as the incidence of females masculinized per site (percent of total females). Anal fin indices greater than two standard deviations (SD) from the mean anal fin index for the reference or upstream (unexposed) site were determined to be masculinized. Standard statistical analyses indicate that the mean ±2 SD would include 95% of all animals and fin indices for the reference population. Therefore, anal fin indices greater than two standard deviations from the reference mean could be considered different or masculinized.

Homogeneity of variance was evaluated using standard $F$ statistic procedures (i.e., Bartlett’s and Levine’s procedures). Anal fin morphology within sex was analyzed using two-way analysis of covariance (ANCOVA) to test for significant variation by site and year. Site differences within year were also analyzed by one-way analysis of variance (ANOVA). Significant differences in the ANCOVA and ANOVA were followed by multiple comparison tests using Tukey’s HSD. Within site, differences between years were analyzed by Student’s $t$-test. Statistical significance was set at $\alpha < 0.05$ for all tests. All statistical analyses were conducted using SAS version 9.0.

Fig. 2. Map of Rice Creek and the St. Johns River, U.S.A. A) Relative location in Florida. B) Field collection sites on Rice Creek. *Gambusia* (mosquitofish) were collected each sampling year at: Upstream Site, Discharge Site, and Downstream Site.
Adult male gonopodia were not influenced by effluent exposures and differences between sites and year were not detected (Fig. 4). Gonopodia were marked by terminal differentiations (hooks, serrae, and blade; data not shown). Photos of normal male and female Gambusia as well as a representative masculinized female are summarized in Fig. 3. Terminal structures signify complete maturation and gonopodial development. Male anal fin indices were always larger than those determined for females, as expected, across all sites and years.

Major process changes at the mill in 2001 were associated with a reduction, but not elimination, in the masculinization response for female mosquitofish (Fig. 5). Dose-dependence was implied by a decreasing elongation with an increasing distance from the discharge point. However, there was no clear dose-dependent response pattern detected since masculinization responses were often times similar across both downstream sites, which might also suggest a threshold response at high exposure sites. Reductions in this masculinization response were observed gradually across 1999 through 2009, with an absence of response in female Gambusia during 2004, 2006, and 2008 to 2009. These data indicate that pulp and paper mill upgrades and process modifications have resulted in the elimination of this biological response in Gambusia.

In addition to mean tendencies for anal fin indices, the percent of the total females collected per site per year were also analyzed for the incidence of masculinization (mean greater than two standard deviations from upstream/unexposed site). Results (Fig. 6) demonstrate that the incidence of masculinized females mean anal fin index for each site was declining across years. Indeed, the masculinization response is not consistent across females within a population, but rather a range of responses within a population, such that each population includes non-masculinized and differing degrees of masculinized females.

Discussion and Conclusions

An anal fin index (ratio of length of rays 4/6) was utilized to assess relative anal fin length or masculinization. Significant differences were noted, with normal gonopodia observed in males, as expected, for Gambusia across all sites and years.

In 2001, the mill began a series of long-term mill improvements and/or major upgrades and modifications...
Fig. 4. Male *Gambusia*. Index of anal fin elongation or masculinization (linear ray 4/ray 6) for each site and year (mean ± SE). No differences noted between sites within each year.

Fig. 5. Female mosquitofish (*Gambusia*). Index of anal fin elongation (linear ray 4/ray 6) for each site and year (mean ± SE). Asterisks indicate statistically significant differences within year as compared with the upstream site (p < 0.05).
that were conducted or implemented through 2008, and that are summarized above in the introduction. However, this study was not designed to delineate cause-and-effect relationships between specific process modifications and process upgrades, or changes in discharge chemical constituents. Indeed, many process changes were underway simultaneously during a given year and the implementation of such process changes often resulted in dual exposure differences for *Gambusia*. For example, Cluster-Rule compliance during 2001 involved both the primary goal of bleaching process upgrades to elemental chlorine free procedures as well as improved black liquor recovery, water use reductions, and condensate stripping, which occurred at multiple points during 2001 through 2008. The efficient upgrade of the mill involved many significant process upgrades and modifications that could simply not be separated into discrete events. Therefore, it was not possible in this study to determine what specific mill process change modifications resulted in diminished responses. Nonetheless, the primary bleaching process modifications in 2001 did not result in a diminished masculinization response, while the subsequent process modifications during 2002 through 2008, which focused on brown-side processes, black liquor recovery, and water conservation, did collectively result in the diminished masculinization responses reported in this study. These data therefore suggest that the brown-side, plant-derived chemical components are those most likely responsible for masculinization of *Gambusia*.

Cluster Rule compliance in 2001 did not eliminate or reduce the masculinization response in wild female mosquitofish inhabiting Rice Creek. However, subsequent reductions in this response were observed in 2004 following a series of reductions in black liquor loss and improvements in the brown-side of mill operations. The return of a significant masculinization response in 2005 may have been linked to a failure of the mill primary clarifier during the spring of 2005 prior to sample collections. However, these results do not indicate a specific relationship between masculinization and specific chemical components or mill processes, as the study did not enable such delineations of cause-and-effect relationships. Nonetheless, these results clearly demonstrate that the culmination of the process changes at the Palatka mill from 2001 to 2006 resulted in the elimination of the masculinization response in female mosquitofish inhabiting Rice Creek. However, these data do not indicate the specific process changes that may have altered masculinization responses. Although process changes and upgrades implemented since 2006 likely resulted in significant discharge improvements and water conservation, the data did not indicate additional effects on masculinization responses in *Gambusia*.

Historic data (Howell et al. 1980; Bortone and Drysdale 1981; Cody and Bortone 1997) prior to these studies had indicated masculinization responses in female *Gambusia* exposed to pulp and paper discharges that resulted in near-gonopodial formation in females. However, the results of the current study indicated that significant reductions in this response parameter had occurred by the initiation of these studies in 1999 to levels which were often times at minimal statistical detection limits; these reductions in response were likely due to other long-term mill upgrades prior to 1999 and/
or the masculinization response at Rice Creek being less than previously suggested. These original reports of masculinization in Gambusia speculated that such responses would result in diminished reproductive success. Although the current study did not evaluate reproductive success as a function of mill upgrades and process modifications, a study assessing reproductive success at multiple Florida receiving streams was conducted (see Noggle et al. 2010).

The concentrations of bioactive compounds in Rice Creek and exposed fish were not determined in this study, but data suggest a relationship between brown-side (not bleaching related) mill chemistry and the masculinization response. Wood extractives present in final effluent can vary widely over short periods of time. The Rice Creek mill cycles between hardwood and softwood tree species, and softwoods generally contain more wood extractives such as phytosterols (Smook 1999; Svenson and Allard 2004). In addition, there is the “black box” of bacterial communities, and how they change over time in the predischARGE secondary treatment lagoons and the receiving stream are unknown. Even more exposure complexity may occur in these low-flow systems from precipitation and periods of drought and flood. A scenario of dynamic exposure appears plausible. Unfortunately, lacking exposure data specifically for these studies limited our ability to evaluate this concept of dynamic effluent exposure and a complete demonstration of improved effluent quality due to process changes.

Evaluations of effects on other fish species has been conducted for the Rice Creek system using controlled effluent exposures, providing indirect information on exposure before and after process changes (Sepulveda et al. 2001, 2003). Life cycle exposure to fathead minnows by NCASI (National Council for Air and Stream Improvement) occurred in 1998, 2002, 2003, and 2008 (NCASI 2000; NCASI unpublished data). Reproductive biomarker assessments for largemouth bass were also conducted in 1998, 1999, 2000, 2001, 2003, 2005, and 2008 (Sepulveda et al. 2001, 2003; Gross TS unpublished data). Both studies indicated significant reductions in responses to mill discharge with process modifications and upgrades as well. Based upon limited chemical analyses of 100% whole effluent samples, before process changes this mill had some of the highest concentrations of organic compounds compared with other kraft mills studied by NCASI. For example, 615 ± 369 μg/L for three fatty acids, 4,008 ± 1,675 μg/L for nine resin acids, 174 ± 33 μg/L for three chlorinated resin acids, and 380 ± 169 μg/L for four phytosterols. These concentrations dropped substantially after process improvements: chlorinated resin acids dropped by 97% while resin acids, fatty acids, and phytosterols dropped an average of 80%. The fact that we observed a relatively modest reduction in effects in the face of these significant reductions to effluent components further supports the concept of a threshold effect as opposed to a dose-response effect. Virtual removal of chlorinated compounds also implies they are not the bioactive agents causing anal fin elongation in mosquitofish.

In addition to the responses reported in female Gambusia, early reports also suggested that precocious maturation in male fish may also be occurring in response to mill discharge exposure (Howell et al. 1980; Bortone and Drysdale 1981). The current study did not evaluate precocious maturation in male Gambusia. However, analysis of body size as an indicator of age did not indicate any site or exposure differences and therefore suggested that this response was also likely eliminated by process improvements since 2001.

Although the exact process improvements and upgrades responsible for the elimination of the masculinized response in Gambusia could not be determined in the current study design, the results clearly demonstrate that pulp and paper mill upgrades and process modifications do result in significantly reduced responses of species exposed in discharge locations. The implications of these diminished responses on Gambusia populations were not evaluated in the current study. However, robust populations were present at both reference and exposed sites throughout these study efforts.

References


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Reproductive Success of Eastern Mosquitofish (Gambusia affinis) Exposed to Pulp and Paper Dominated Receiving Streams and Effects of Masculinization Responses

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Mosquitofish (Gambusia) are sexually dimorphic; adult males have an elongation of the anal fin to form a copulatory structure known as a gonopodium. Several studies since the early 1980s have reported elongated anal fins in female mosquitofish exposed to pulp and paper mill discharge, a phenomenon known as masculinization. Although adverse impacts have been suggested in these previous reports, the influence of masculinization on reproductive success has not been assessed for pulp mill effluent-exposed eastern mosquitofish (Gambusia holbrooki or affinis). The current study compared fecundity (number of fry per female at parturition) to an index of masculinization (ratio of anal fin rays 4 to 6). Pregnant females were collected from two effluent-receiving streams in Florida: Rice Creek and the Fenholloway River, over two reproductive seasons (2003 and 2004). Masculinization was consistent between years, with clear effects at the Fenholloway River site, while the response was minimal or nondetectable at the Rice Creek site. Masculinization was not correlated with the production of fry/fecundity at either site. Data suggest differing reproductive seasonal strategies between basins and populations but do not demonstrate any effects of pulp and paper exposure on reproductive success in mosquitofish.

Key words: masculinization, reproductive success, Gambusia, pulp and paper, fish

Introduction

Sublethal effects of paper mill effluent exposure on fish have been a major focus of aquatic environmental health research for the past two decades (Sodergren 1991; Servos et al. 1996; Ruoppa et al. 2000; Suthridge et al. 2003; Borton et al. 2004). Reported effects include induction of liver detoxification systems, alterations in sex steroid concentrations and production/metabolism, reduced gonadal development, and decreased egg production (Van der Kraak et al. 1992; Gagnon et al. 1994; Munkittrick et al. 1999; NCASI 2000; Sepulveda et al. 2001, 2003; McMaster et al. 2003; Parrott et al. 2004). Whether or not these effects represent actual adverse effects in terms of reproductive success or population and community level impacts have remained largely untested.

Development of male-like secondary sex characteristics in female Gambusia, specifically masculinization of the anal fin into a gonopodial-like structure, has been historically reported in mill effluent-receiving streams (Howell et al. 1980; Drysdale and Bortone 1989; Cody and Bortone 1997; Bortone and Cody 1999; Jenkins et al. 2001; Parks et al. 2001). Although negative impacts on reproduction were initially implied or suggested by this phenomenon, normal ovaries lacking any testicular tissue were consistently reported in masculinized females (Howell et al. 1980; Hunsinger et al. 1988; Ellis et al. 2003; McCarthy et al. 2004). In addition, sex ratios of Gambusia reared in 100% final effluent were not altered from controls (McCarthy et al. 2004). Decreased potential fecundity, measured as brood size of developing embryos, was reported in early preliminary work (Rosa-Molinar and Williams 1984); however, these observations were not detected in more recent studies (Felder et al. 1998; D’Surney et al. 2000). Recent reports (Noggle 2005; Noggle et al. 2010) have also demonstrated that improvements in processing technologies and reductions in black liquor losses by the paper industry have reduced masculinization responses in Gambusia relative to these initial investigations: (Howell et al. 1980; Drysdale and Bortone 1989; Cody and Bortone 1997).

Gambusia, as members of the live-bearing family Poeciliidae, develop eggs internally and ovulate immediately before parturition of fry (Meffe and Snelson 1989). As nonsuperfetating lecithotrophes, Gambusia develop a single brood at a time and exhibit yolk loading of eggs similar to egg-laying (oviparous) species without maternal investment during embryological development (Turner 1937). Compared to egg-laying species, reproduction is asynchronous and the reproductive season occurs across summer months with low to no reproduction in winter months (Constanz 1989). Environmental cues control the beginning and end of the reproductive season: the onset of the reproductive season is triggered by a rise in water temperature, while photoperiod (decreasing day length) controls gonadal recrudescence (Koya and Kamiya 2000;
Koya and Iwase 2004). This complicates the study of the effects of environmental toxicants on fecundity in this species.

A major drawback among existing studies of Gambusia and reproductive function in paper mill effluents is the lack of corresponding anal fin morphology data to evaluate the potential association, if any, between masculinization and reproduction (i.e., fecundity measured by brood size). Moreover, as far as we are aware, no research has been conducted to assess measures of fecundity across populations within a water basin (a measure of fecundity and recruitment) for Gambusia. The objective for the current study was to evaluate fry production in female Gambusia collected from effluent-dominated receiving streams, and evaluate whether masculinization alters reproductive success or function.

**Materials and Methods**

**Mill Characteristics**

The Fenholloway River and Rice Creek mills are very different in wood furnish, processing, and product. The Rice Creek mill, in operation since 1947, is a bleached/unbleached kraft mill that produces kraft and tissue papers. Furnish is 50:50 hardwood:softwood. The Fenholloway River mill, in operation since 1954, is a dissolving kraft mill that produces high grade cellulose. Furnish is 100% softwood. At the time of fish collections, both mills used aeration with microbial degradation as secondary effluent treatment. In addition, Rice Creek had activated sludge. Effluent discharge volumes at the time of collections were 106 million litres per day (mld) (28 million gallons per day [mgd]) into Rice Creek and 163 mld (43 mgd) into the Fenholloway River.

**Site Locations**

Mosquitofish were sampled during 2003 and 2004 downstream from two paper mill effluent-dominant streams in Florida where masculinized females have been previously documented (Fig. 1). The effluent-dominated streams were: the Fenholloway River associated with the Buckeye Technologies, Perry, Fla. mill; and Rice Creek associated with the Georgia Pacific, Palatka, Fla. mill. One in-stream exposed and one unexposed site from each of these two systems were surveyed for fry.

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**Fig. 1.** Maps of field sites. A) Relative location of the stream systems in Florida. B) Fenholloway River sites. C) Rice Creek sites. Site symbols distinguish exposed (effluent exposure) as dark circles, while unexposed (reference or upstream) sites are represented by dark stars.
Reproductive Success of Masculinized Mosquitofish

production in females. Within Rice Creek, mosquitofish were collected from a site 8 km upstream of the effluent discharge (unexposed = RC-Upstream) and from the discharge point (exposed site = RC-Exposed). Within the Fenholloway River, fish were collected as close as possible to the discharge point which was 5 km downstream of the effluent discharge (exposed = FR-Exposed), and at a reference site several km upstream from the mouth of the Econfina River (unexposed = FR-Ref).

Fish Collections

Mosquitofish were collected at each site at one time point in 2003 to validate procedures and produce preliminary results. Fish were collected monthly for four months during 2004 to better assess reproductive success across the primary reproductive season. Dip nets and multiple personnel were used to collect mosquitofish within one day in each system. Sampling concluded at each site when an estimated 75 to 100 adult female *Gambusia* were collected for fry production studies. All *Gambusia* were kept alive in aerated bait buckets and transported back to the laboratory for processing. Water quality parameters were measured at each site at the time of mosquitofish collection: dissolved oxygen, temperature, pH, conductivity, salinity, and turbidity.

In the laboratory, *Gambusia* were sorted into age-sex groups, giving preference to gravid females for the preparation of fry production studies. Fish were handled as little as possible using latex gloves to minimize stress on gravid females. Groups were divided as follows: gravid females (gravid spot and swollen abdomen); nongravid females (lack of or partial gravid spot and slim abdomen); adult males (fully differentiated gonopodium); developing males (elongated gonopodium lacking terminal differentiations); and juveniles (<20 mm standard length and lacking gravid spot and gonopodium). Urogenital papillae were used to confirm gender when necessary using a dissecting microscope. Females were evaluated for masculinization using a standard anal fin index which represents the ratio of length for fin rays 4 and 6 (Fig. 2).

Laboratory Fry Production

Approximately fifty gravid females from each site (total 4 sites) were held for thirty days to monitor fry production for each of the 2003 and 2004 collections. Each female was placed individually in a modified plastic hatchery chamber, purchased from Aquatic Ecosystems (Apopka, Fla.), that included a hinged lid to prevent escape and a 7.6 cm length of artificial green *Cabomba* grass to provide cover for females and fry. Upper portions of the

**Fig. 2.** Anal fin morphology for adult *Gambusia*. Females are generally larger in body weight and length than male fish. Male fish have an elongated anal fin, representing an elongation of fin rays 3, 4, and 5, to form a copulatory structure known as a gonopodium. Females normally have no or minimal elongation of these fin rays. An anal fin index, or ratio of length for fin rays 4 and 6, compares the primary elongated fin ray with a fin ray which does not elongate to calculate an index of anal fin elongation or masculinization.
hatchery chamber were available to females, while the lower portion was accessible only by fry via a slotted barrier. Newborn fry instinctively seek escape and protection from the mother due to her cannibalistic instinct. Fish acclimated to a 50:50 pond-well water mix for 24 to 48 hours. After acclimation, hatchery chambers with females were transferred to two 1.2-m by 2.4-m by 15-cm (4' by 8' by 6'') shallow tanks receiving a 50:50 filtered pond-well water mix. Chambers were randomized with respect to location in tanks, and 100 chambers filled each tank allowing for up to 200 chambers total. Full spectrum lighting was set on a 14:10 hour light:dark schedule to simulate increased photoperiod and keep females in reproductive mode.

Chambers were monitored daily for fry production. Fry were removed immediately when detected to be euthanized, counted, and preserved in 10% neutral buffered formalin for assessment of deformities and fry weight. Chambers were rinsed and females were returned to the tank for observation of secondary brood production. Females were fed ad libitum with Tropical Prime flakes (Zeigler Brothers, Gardners, Pa., nutritional composition 45% minimum protein, 9% minimum fat, 4% maximum fibre). Water quality (dissolved oxygen, temperature, pH, conductivity, salinity, incident light, and turbidity) was measured three times a week. Incident light was measured at 15 points evenly spaced throughout each tank. All other water quality measurements were made at a single location in each tank.

Measurements and Statistics

Body weight and standard length were used to calculate condition factor, $K = \frac{\text{weight}}{\text{length}^3} \times 100$ (g/cm$^3$), as an indication of overall health used by the aquaculture industry (values of at least 1 are considered healthy, Hile 1936). For fry production studies, increased body length has been shown to positively affect brood size (Krumholz 1948; Hughes 1985; Meffe and Snelson 1989), so the number of fry produced by each female was divided by her standard length for statistical analysis. Fry production data were analyzed between sites using $t$-tests within systems. Any data failing tests for normality and homogeneity of variance were transformed using log transformations. Interactions by site and month and anal fin index (masculinization) were also analyzed using an analysis of covariance (ANCOVA), and influence of month and anal fin index within each site were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s Honestly Significant Differences (HSD). Statistical significance was set at $\alpha = 0.05$ for all tests. All statistical analyses were conducted using SAS version 9.0.

The length ratio of anal fin rays 4 and 6 was calculated as a sensitive index of anal fin elongation (Angus et al. 2001; Noggle 2005). Fin morphology masculinization was analyzed within sex using one-way ANOVA to test for significant variation by site. Any data failing tests for normality and homogeneity of variance were transformed using ANOVA were analyzed for multiple comparisons using Tukey’s HSD.

Since water parameters were measured repeatedly, water quality (2004) and chemistry (2003 and 2004) were analyzed using one-way ANOVA to test for significant variation by site (t-test for Rice Creek samples). Significant differences using ANOVA were also analyzed for multiple comparisons using Tukey’s HSD.

Results

Water Quality

In general, most water quality parameters (temperature, conductivity, salinity, and turbidity) were higher at effluent-exposed sites compared with unexposed sites (Table 1). The pH did not differ across sites. Dissolved oxygen was lower at the Fenholloway River exposure site during 2003, but not during 2004. Elevated temperature at effluent-exposed sites may be important for differences in reproductive stage since reproduction is initiated by a rise in temperature (Koya and Kamiya 2000).

In the fry production tanks, conductivity, salinity, and turbidity were low and comparable to the reference and upstream field sites (Table 2). Temperatures were intermediate between exposed and unexposed sites,

<table>
<thead>
<tr>
<th>Site</th>
<th>Fenholloway River</th>
<th>Rice Creek</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reference</td>
<td>Exposed</td>
</tr>
<tr>
<td>Temperature ($^\circ$C)</td>
<td>22.8 ± 0.6</td>
<td>28.4 ± 1.0</td>
</tr>
<tr>
<td>Conductivity (µS)</td>
<td>287.4 ± 69.4</td>
<td>2,105 ± 211.3</td>
</tr>
<tr>
<td>Salinity (ppt)</td>
<td>0.2 ± 0.03</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Dissolved Oxygen (mg/L)</td>
<td>4.50 ± 0.27</td>
<td>4.92 ± 1.42</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>1.84 ± 0.27</td>
<td>13.43 ± 1.42</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 ± 0.2</td>
<td>7.3 ± 0.2</td>
</tr>
</tbody>
</table>

TABLE 1. Mean water quality parameters (mean ± SE) at field sites where female mosquito fish were collected for fry production studies during 2003–2004. All parameters were statistically different between exposed and unexposed sites within each system.
averaging closer to exposed sites. Dissolved oxygen was adequate for fish survival (overall average 6.34 mg/L). Incident light was consistent throughout the tanks.

**Gambusia Morphology**

Body size was similar for female *Gambusia* across sites and years. Mean body weight was 0.504 ± 0.058 g, while standard body length was 29.8 ± 0.96 mm. Condition factor was above one for all collections and sites (1.91 ± 0.04), which indicated adequate general health across all sites.

**Fin Morphology**

Anal fin elongation in female *Gambusia* was detected only in the Fenholloway River exposure site in 2003 and 2004 (Tables 2 and 3). The masculinization response was not detected at the Rice Creek exposure site in 2003 or 2004. This absence represents the first time since 1998 (when the response was initially monitored at Rice Creek) that masculinization was not detected. The diminished masculinization response at Rice Creek may have resulted from a cumulative effect of process changes and upgrades at the Georgia Pacific Palatka mill since 1998 (Noggle et al. 2010).

**Fry Production**

A preliminary assessment of reproductive success and fry production for *Gambusia* as a function of mill exposure was conducted in 2003 to develop and validate husbandry techniques. The results for 2003 (Table 3) demonstrated the utility of using hatchery chambers and captive husbandry procedures to assess both reproductive success and fry production for *Gambusia* from natural field sites in the laboratory. Approximately 90% of all females subsequently produced fry under captive conditions, and approximately 18% of all females produced a secondary clutch at a mean interbrood interval of approximately 25 days. Fry weights were similar across sites during 2003 (Table 3) and the incidence of fry anomalies or deformities

### TABLE 2. Water quality parameters measured three times weekly during laboratory fry production of female mosquito-fish collected from field sites in Rice Creek during summer 2003 to 2004

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>25.2 ± 0.24</td>
<td>24 - 27</td>
</tr>
<tr>
<td>Conductivity (μS)</td>
<td>292.6 ± 10.8</td>
<td>320 - 335</td>
</tr>
<tr>
<td>Salinity (ppt)</td>
<td>0.14 ± 0.01</td>
<td>0.1 - 0.2</td>
</tr>
<tr>
<td>Dissolved Oxygen (mg/L)</td>
<td>6.34 ± 0.27</td>
<td>5.1 - 7.8</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>0.77 ± 0.25</td>
<td>0.2 - 1.4</td>
</tr>
<tr>
<td>pH</td>
<td>8.0 ± 0.06</td>
<td>7.5 - 8.8</td>
</tr>
<tr>
<td>Incident light (μmol photons/s/m²)</td>
<td>12.6 ± 0.1</td>
<td>12.1 - 12.9</td>
</tr>
</tbody>
</table>

*a Measured once a week.

*b At 400-700 nm.

### TABLE 3. Reproductive and morphological characteristics of females (♀) collected from Fenholloway River and Rice Creek and monitored for fry production in 2003

<table>
<thead>
<tr>
<th></th>
<th>Fenholloway River</th>
<th>Rice Creek</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>FR-Ref</td>
<td>FR-Exposed</td>
</tr>
<tr>
<td>♀ Start</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Standard Length (mm)</td>
<td>30.9 ± 0.52</td>
<td>28.2 ± 0.57</td>
</tr>
<tr>
<td>Index of Anal Fin Elongation (Ray 4/Ray 6)</td>
<td>1.2 ± 0.01</td>
<td>1.5 ± 0.06</td>
</tr>
<tr>
<td>% ♀ Parturition b</td>
<td>91</td>
<td>92</td>
</tr>
<tr>
<td>Total Fry (1° production)</td>
<td>689</td>
<td>531</td>
</tr>
<tr>
<td>1° Adjusted Fecundity (fry/female)</td>
<td>19.8 ± 2.1</td>
<td>15.1 ± 3.3</td>
</tr>
<tr>
<td>1° Fry Weight (mg) a</td>
<td>8.9 ± 0.21</td>
<td>8.5 ± 0.24</td>
</tr>
<tr>
<td>% ♀ with 2° Production c</td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td>Total fry (2° production)</td>
<td>47</td>
<td>129</td>
</tr>
<tr>
<td>2° Adjusted fecundity (fry/female)</td>
<td>19.7 ± 1.6</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td>2° Fry weight (mg) a</td>
<td>9.4 ± 0.25</td>
<td>7.7 ± 0.26</td>
</tr>
</tbody>
</table>

| Median interbrood interval (days) | 24 | 24 | 25 | 27 |

*a Mean ± SE.

*b Referring to primary (1°) production of surviving females.

*c Referring to secondary (2°) production of females that had primary production.

*d Significantly different than REF site (p < 0.05) indicating masculinization.

*e Significantly different than the unexposed site within basin.
was generally 5% or less across all sites. Observed deformities included edema, skeletal abnormalities (lordosis and scoliosis), or premature abortion of embryos. Site comparisons suggested both a decreased total fry production per site for effluent-exposed sites versus nonexposed sites within both basins (Table 3), as well as a reduced adjusted fecundity (Fig. 3; mean fry per female as a function of body weight) for exposed sites. However, females were collected in a single month during a several month period of the summer reproductive season for Gambusia in Florida. Thus, the study design did not account for potential asynchronous reproductive activity across sites. Additional analysis accounting for such variance was necessary to further verify any effects of mill exposure on Gambusia reproductive success.

To account for asynchrony of Gambusia reproduction across sites, fry production was also evaluated across four months (May, June, July, and August) of the 2004 summer reproductive season for Gambusia collected from the Rice Creek and Fenholloway River basins. Results for 2004 (Table 4) indicated significant differences across months for percent of females producing offspring, total fry produced, and adjusted fecundity for both basins, as well as site differences within each basin. While masculinization was consistently observed for adult females at all summer months from the exposed site within the Fenholloway basin, there was no detected correlation between masculinization as measured by the anal fin index and reproductive success in Gambusia. The percent of total females producing fry, the total number of fry produced, and adjusted fecundity were not associated with anal fin elongation/masculinization. Results for adjusted fecundity across sites and months did, however, indicate significant seasonal differences between the Rice Creek and Fenholloway River basins (Fig. 3), implying potentially differing reproductive strategies rather than masculinization effects. Indeed, the presence of fin elongation was not representative of fecundity, suggesting that anal fin elongation was not predictive of any observed effects on reproduction.

**Discussion and Conclusions**

In general, most adult Gambusia females produced fry regardless of pulp and paper effluent exposure. Primary clutch sizes were similar across sites and within basins, although the number of offspring per female varied widely from a few fry to several dozen. These results were within ranges reported historically for Gambusia (Krumholz 1948; Rosen and Bailey 1963; Hughes 1985; Meffe and Snelson 1989; Specziar 2004).

Although Gambusia are considered nonsuperfetating, a Bahamian species (Gambusia hubbsi) demonstrated superfetation as a possible tool to reduce reproductive costs (Downhower et al. 2002). Superfetating species produce small broods (around 1 to 5 fry) more frequently than nonsuperfetating species, and a shift to this tactic could potentially bias comparison to nonsuperfetating populations. Therefore, this trait was examined for potential alteration by mill effluents by retaining females for the full 30 days even after primary production in 2003. Fifteen to thirty percent of primary producing females also produced a second clutch of comparable size within the established 24 to 28 day interbrood interval for nonsuperfetation in this species. Thus, effluent exposure did not alter this reproductive strategy. Further, sperm storage by female mosquitofish was reaffirmed since females were not exposed to males during this monitoring and interbrood period.

These studies represent the first examination of actual fry production as a measure of reproductive success in Gambusia exposed to paper mill effluents.

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**Fig. 3.** Summary of results for 2004: adjusted fecundity (mean fry per female) and the percent of total females that successfully produce fry. Rice Creek basin sites have increased fecundity and reduced % females reproducing as compared with the Fenholloway River basin. Data suggest potentially differing reproductive strategies between basins to produce adequate fry for eventual recruitment. Sites within basins do not differ regardless of mill exposure.
Reproductive Success of Masculinized Mosquitofish

Previous investigations of reproduction in this species under effluent exposure were confined to brood size of developing embryos or histological evaluation of gonads, neither of which detected obvious adverse impacts. Further, the current studies are the first to assess masculinization and the relationship to reproductive outcome. Results indicated that reproductive success of Gambusia is likely not impacted by effluent exposure from modern paper mills. Rather, seasonal differences in fecundities have resulted in the adaptation of different site-specific reproductive strategies. Fenholloway River females displayed reduced fecundity and masculinized anal fins consistently over the observation period but with a greater percent of total females successfully reproducing. In contrast, Rice Creek females were more fecund but with a lower percent of total females successfully reproducing. These data emphasize the need to examine seasonal synchrony between sites and other aspects of reproductive strategies for a population as an integral part of assessments for potential adverse effects of exposure to enable the accurate identification of effects. It is also possible that effluent-exposed females begin the reproductive season earlier than females living in nonexposed sites, perhaps caused by higher water temperature rather than differing reproductive strategies across basins. Increased temperature strongly triggers onset of the reproductive season (Koya and Kamiya 2000; Koya and Iwase 2004), and has been associated with an overall increase in reproductive output (Vondracek et al. 1988).

Differences in seasonal reproductive patterns have been described for Gambusia populations living in unexposed conditions under the influence of different predation and food availability (Vondracek et al. 1988; Downhower et al. 2000). Further, Downhower et al. (2000) detected rapid phenotypic adjustment or plasticity in reproductive strategies for populations introduced to predator-free habitats in less than 20 years. Therefore, ecological differences among sites caused by long-term effluent dominance could also affect fecundity. For example, increased turbidity at effluent-dominated sites may decrease predation risk of Gambusia, and eutrophication of effluent-receiving systems may increase food availability as well. The combined effect of these types of ecological factors likely alters reproductive investments and strategies, which may explain observed variation in fecundity between basins in this study. Variation in fecundity over the 2004 reproductive season within a site also supports the concept of two separate reproducing populations: overwintering and young-of-year females (Hughes 1985; Haynes and Cashner 1995; Fernández-Delgado and Rossomanno 1997). This possibility reinforces the importance of documenting reproduction throughout the reproductive season in studies of this nature.

### TABLE 4. Reproductive and morphological characteristics of females (♀) collected for fry production from the Fenholloway River and Rice Creek in 2004

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td># ♂ Start</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>42</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>Standard Length (mm)</td>
<td>31.3 ± 0.3</td>
<td>26.7 ± 0.5</td>
<td>28.5 ± 0.4</td>
<td>28.2 ± 0.5</td>
<td>30.1 ± 0.4</td>
<td>28.8 ± 0.8</td>
<td>24.7 ± 0.6</td>
<td>24.3 ± 0.4</td>
</tr>
<tr>
<td>Index of anal fin elongation (Ray 4/Ray 6)³</td>
<td>1.2 ± 0.01</td>
<td>1.1 ± 0.01</td>
<td>1.1 ± 0.01</td>
<td>1.1 ± 0.01</td>
<td>1.5 ± 0.03</td>
<td>1.6 ± 0.04</td>
<td>1.5 ± 0.04</td>
<td>1.4 ± 0.03</td>
</tr>
<tr>
<td>%♀ Parturition</td>
<td>38</td>
<td>66</td>
<td>80</td>
<td>67</td>
<td>74</td>
<td>68</td>
<td>66</td>
<td>67</td>
</tr>
<tr>
<td>Total fry (1st Production)</td>
<td>100</td>
<td>92</td>
<td>233</td>
<td>135</td>
<td>264</td>
<td>174</td>
<td>221</td>
<td>241</td>
</tr>
<tr>
<td>Adjusted fecundity (fry/female)⁴</td>
<td>3.1 ± 1.0</td>
<td>2.3 ± 0.7</td>
<td>5.2 ± 1.2</td>
<td>4.2 ± 0.9</td>
<td>5.1 ± 0.8</td>
<td>4.3 ± 0.9</td>
<td>5.2 ± 1.0</td>
<td>5.8 ± 0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Upstream May</th>
<th>Upstream June</th>
<th>Upstream July</th>
<th>Upstream Aug</th>
<th>Exposed May</th>
<th>Exposed June</th>
<th>Exposed July</th>
<th>Exposed Aug</th>
</tr>
</thead>
<tbody>
<tr>
<td># ♂ Start</td>
<td>50</td>
<td>49</td>
<td>50</td>
<td>35</td>
<td>50</td>
<td>50</td>
<td>34</td>
<td>49</td>
</tr>
<tr>
<td>Standard Length (mm)</td>
<td>28.6 ± 0.4</td>
<td>30.2 ± 0.5</td>
<td>30.0 ± 0.7</td>
<td>29.4 ± 1.0</td>
<td>28.4 ± 0.4</td>
<td>28.6 ± 0.5</td>
<td>29.6 ± 0.6</td>
<td>31.9 ± 0.5</td>
</tr>
<tr>
<td>Index of anal fin elongation (Ray 4/Ray 6)³</td>
<td>1.2 ± 0.01</td>
<td>1.2 ± 0.01</td>
<td>1.1 ± 0.02</td>
<td>1.1 ± 0.02</td>
<td>1.2 ± 0.05</td>
<td>1.2 ± 0.04</td>
<td>1.2 ± 0.05</td>
<td>1.3 ± 0.05</td>
</tr>
<tr>
<td>%♀ Parturition</td>
<td>4</td>
<td>67</td>
<td>68</td>
<td>57</td>
<td>28</td>
<td>74</td>
<td>62</td>
<td>71</td>
</tr>
<tr>
<td>Total fry (1st Production)</td>
<td>20</td>
<td>155</td>
<td>627</td>
<td>217</td>
<td>74</td>
<td>141</td>
<td>141</td>
<td>541</td>
</tr>
<tr>
<td>Adjusted fecundity (fry/female)⁴</td>
<td>1.2 ± 0.4</td>
<td>2.7 ± 0.6</td>
<td>15.2 ± 1.1</td>
<td>7.8 ± 1.0</td>
<td>1.8 ± 0.5</td>
<td>2.5 ± 0.6</td>
<td>4.8 ± 1.1</td>
<td>14.8 ± 1.0</td>
</tr>
</tbody>
</table>

*Mean ± SE.
*Referring to primary (1st) production of all females.
*Significantly different than unexposed site indicating masculinization.
*Significantly different from unexposed site within month collected.
Decisions on reproductive success based only on fecundity may likely misrepresent actual population patterns; therefore, we recommend documentation of onset and cessation of reproduction across sites. Ideally, population-level studies investigating energetic investments in reproduction would be included to address potential variation in reproductive strategies at effluent-exposed sites. It is possible an earlier onset of reproduction in effluent-exposed fish at the FenHolloway River may counteract the somewhat lower fecundities than the Rice Creek fish exhibited in these studies. Preliminary relative abundance data (Noggle 2005) indicated a greatly increased density at the downstream FenHolloway site in early summer 2003 (May), so an earlier onset of reproduction is tentatively supported. Also, the overall reduced fecundities between years suggested additional environmental factors may be negatively influencing fry production.

The biological relevance of using anal fin length as a bioindicator of effects on reproductive success in Gambusia was weakened by initial reproductive success studies that could not link the observation with altered fecundity. Differences in fecundity may ultimately reflect an adaptation of reproductive strategy in effluent-exposed fish, as opposed to negative impacts on reproductive success. In addition, considering the reduction and in some cases elimination of anal fin elongation in female Gambusia exposed to pulp and paper mill discharge over the past decade (Noggle et al. 2010), mosquitofish masculinization may no longer be a useful marker of adverse effects from exposure to pulp and paper mill effluents.

Future studies would be valuable at the population or fish community level. This study did not account for ecological factors such as predation or eutrophication that may have influenced the observed differences in fecundity. Therefore, we cannot conclude that mosquitofish populations living in effluent-exposed streams are either compromised or enhanced by ecological conditions. However, preliminary relative abundance data for age/sex structure in Noggle (2005) did not suggest adverse population structures at effluent-exposed sites.

References


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Reproductive Steroid Responses in Fish Exposed to Pulp Mill Condensates: An Investigation of Cause Case Study

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An investigation of cause (IOC) approach integrating artificial stream exposures and laboratory bioassays has been used to identify waste stream sources of contaminants at the Irving Pulp & Paper Ltd. mill, in Saint John, New Brunswick, Canada. Chemical recovery condensates have shown the greatest potential for reducing circulating steroids in mummichog (Fundulus heteroclitus), an endemic fish species. A solid phase extraction (SPE) technique was developed to isolate hormonally active substances from the condensates, and a toxicity identification evaluation approach was used to gain a better understanding of the chemical characteristics of the active substances. Extracts were fractionated by high performance liquid chromatography (HPLC) and the fractions were used in a seven-day bioassay. Dose-response experiments indicated that steroid reductions in male mummichog were observed consistently after a 4% (vol/vol) exposure. At 4% (vol/vol), however, steroid reductions were not observed in fractions of the active SPE extract generated by HPLC. Some fractions actually induced increases in plasma testosterone. Recent work has focused on understanding what methodologies must be used to handle the semivolatile condensates to ensure 100% chemical recovery and retention of biological activity. Results are summarized in the context of developing an industry-wide IOC framework.

Key words: Fundulus heteroclitus, reproductive steroids, condensates, toxicity identification, investigation of cause

Introduction

In Canada, studies conducted as part of the federally-regulated Environmental Effects Monitoring (EEM) program have found a pattern of increased condition, increased liver size, and reduced gonad size (termed metabolic disruption) in wild fish collected from the receiving environment, relative to reference sites (Munkittrick et al. 2002). These effects are presumed to be caused by the presence of endocrine-active contaminants in the effluent. However, despite a great deal of effort, it is unclear what components are responsible. Research on characterizing the causative compounds has been hindered by the complex nature of mill effluents and the variability in sensitivity of different fish species. Cause and effect relationships between the components of the effluent and fish responses are difficult to determine given the range of chemicals present (e.g., wood-derived, production-derived, treatment-derived) and their potential combinations. As well, protocols for only a few fish species have been described that can link responses to wastestream sources and identify causative chemicals (Martel et al. 1997; Parrott and Wood 2002; Bosker et al. 2009a).

Studies conducted at Irving Pulp and Paper Ltd., a bleached kraft pulp mill in Saint John, New Brunswick, Canada, were some of the first to implement an investigation of cause approach (Hewitt et al. 2003) for identifying specific hormonally-active waste streams within the mill (Dubé and MacLatchy 2000a, 2001; Hewitt et al. 2002; Belknap et al. 2006; Shaughnessy et al. 2007). Since 1997, our objectives at this mill have been to: 1) identify the sources within the mill processes of contaminants that cause reproductive responses; 2) characterize the causative compounds within the source waste stream(s); and 3) identify potential technological solutions that improve effluent quality at end-of-pipe. We have approached these objectives in a step-wise fashion that has resulted in improved understanding of the potential of one waste stream (5th effect chemical recovery condensates) to cause reproductive endocrine changes in fish. Here, we review these studies, and highlight new information from our most recent work that will determine future research directions.

Irving Pulp & Paper Ltd. (IPP)

IPP is a bleached kraft pulp mill located at the mouth of the Saint John River in Saint John, New Brunswick, Canada. This mill produces approximately 330,000 tonnes per year of market pulp from hardwood (primarily

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maple and birch) and softwood (primarily spruce, fir, and pine) furnish. It discharges approximately 96,000 m³/day of effluent (treated via reverse osmosis and a moving bed bioreactor as described below) into the Saint John River. The bleaching technology at this mill is elemental chlorine free with a DE₂PDE₅P (D = chlorine dioxide bleaching stage; E = lignin extraction bleaching stage with either oxygen [O] or hydrogen peroxide [P]) bleaching sequence. Prior to discharge, a moving bed bioreactor (installed in 2000 and using thermophilic bacterial action) removes biological oxygen demand (BOD) from two bleach plant effluent streams (D, EfP) prior to being combined with other process wastes in the final discharge. Since the late 1990s, the mill has implemented several process changes, including two-stage brownstock washing and four-stage closed screening, oxygen delignification and two-stage postoxygen washing, improved foul condensate stream stripping and spill recovery, and upgraded reactors.

Until 1998, condensates from the 5th and 6th effect evaporators were recycled as wash water in other areas of the mill. Condensates from the 5th effect evaporator and bleach plant effluents had high Microtox toxicity, BOD, and chemical oxygen demand (Dubé and MacLatchy 2000a). In 1998, a reverse osmosis (RO) system was installed to treat the condensates from the 5th effect evaporator prior to its reuse as wash water within the mill.

RO is a reverse crossflow membrane separation process that is typically used to purify water and remove dissolved salts and small organic particles less than 1 nm in diameter. The technology has been used in the pulp and paper industry to reduce chemical oxygen demand, total organic carbon, and colour of different process effluents. However, the majority of these uses were implemented on a pilot scale to explore feasibility (Knudsen et al. 1996). At IPP, condensates from the 5th effect evaporator (RO feed) are applied under pressure to force it through a semipermeable membrane for fine filtration and reduction of BOD (approximately 4,000 L/min). The RO permeate (clean condensates that have passed through the membrane, approximately 99% of the flow) is then reused in the mill, and ultimately discharged into the main chemical sewer. The retentate (chemicals rejected by the RO membrane, approximately 1% of the flow) is burned in the bark boiler (Dubé and MacLatchy 2000a).

**Investigation of Cause Approach**

Here we define "investigation of cause" (IOC) as a multilayered guidance framework for the identification of the cause of environmental effects (Hewitt et al. 2003). The framework includes tiers to: 1) define whether there is an effect; 2) investigate individual process wastes to determine those contributing to final effluent effects; and 3) characterize and identify the chemical classes involved in causing biological responses. The framework has been constructed around research approaches at different pulp mills; our work at IPP has formed the basis for conducting in-mill waste stream level investigations (Table 1). A modified framework has been developed which provides guidance on conducting investigations relating to contaminant effects (including metabolic disruption) as well as effects due to eutrophication (Hewitt et al. 2005). The development of the current approaches used for in-mill source identification occurred in three distinct phases which are described below.


Our initial work began with three questions: 1) could artificial stream (mesocosm) technology be developed to assess pulp and paper effluent effects for the EEM program to gain a more controlled understanding of the effects of the effluent; 2) could particular waste streams in the pulp mill be linked to fish reproductive responses as a mechanism to isolate where treatment technologies might be best served; and 3) could we use mesocosms to determine the effectiveness of potential treatment technologies? The 1997 study design involved the use of mesocosm systems which systematically exposed mummichog (a small-bodied, endemic saltwater fish, Fundulus heteroclitus, of the local area) to multiple in-mill process wastes (condensates, post-oxygen washer filtrates, and final mill effluent) at environmentally-relevant 1% (vol/vol) concentrations (Dubé and MacLatchy 2000a). In this case, mesocosms were used because 1) traditional field sampling could not be carried out in the confounded estuarine environment; and 2) the system allowed the isolation and simultaneous testing of several waste streams at environmentally-relevant concentrations. It was subsequently determined that both sexes of fish had reduced plasma testosterone levels at 1% (vol/vol) final effluent. Females showed increased liver size and decreased in vitro production of plasma 17β-estradiol following exposure to the condensates, suggesting that the condensates compose an important process stream that causes sublethal responses in mummichog.

In March 1998, IPP installed an RO system on the 5th effect evaporator of the chemical recovery system so that its final effluent could meet acute toxicity and BOD regulations; this provided us the opportunity to continue our IOC studies. After RO installation, mummichog plasma testosterone levels were unaffected following exposure to final effluent (at 1% [vol/vol]), a significant improvement from the previous year (Dubé and MacLatchy 2000a). However, significant reductions in plasma testosterone were observed at 50% (vol/vol), suggesting two possibilities: 1) the RO system reduced, but did not entirely eliminate, endocrine disrupting substances (EDSs) from condensates and/or 2) other sources of EDSs must be present. The results did confirm that chemical recovery condensates may be a source of EDSs in bleached kraft pulp mill effluent, and that RO treatment may be a successful treatment technology to
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*TIE = toxicity identification evaluation.*
improve effluent quality in regard to its effects on fish reproduction (Dubé and MacLatchy 2000a).

In 1999, mummichog were exposed in 7- and 21-day laboratory exposures to RO feed (5th effect condensates; 1% and 3% [vol/vol]), RO permeate (5 and 25% [vol/vol]), bleach plant effluent (1% and 50% [vol/vol]), and combined mill effluent (pulp mill wastest added to tissue mill wastest). The protocol used was a static exposure with daily renewal of water and effluents. Results from this study confirmed that condensates depress plasma testosterone levels in mummichog and confirmed the previous year’s study that RO treatment removed the potential of the condensates to lower plasma testosterone levels in final mill effluent at an environmentally-relevant concentration (Dubé and MacLatchy 2001). Further work subsequently showed that at very high concentrations (25% [vol/vol]) RO permeate did not lower testosterone levels in exposed fish and that additional sources of EDSs may be present in the mill since 50% (vol/vol) final effluent consistently lowered plasma testosterone. Further studies on the endocrine status of laboratory-held mummichog confirmed that the RO feed and RO retentates contain endocrine-disrupting properties while RO permeate does not (MacLatchy et al. 2001).

Since the RO system was originally implemented to address regulatory toxicity and BOD compliance, we have hypothesized that these parameters may be used as “markers” of the noncellulosic organic material considered as waste in pulping operations. This material appears to contain the EDSs, which we have shown when fish were exposed to final effluents when the RO was operated in “on” and “off” modes (Dubé and MacLatchy 2000b) and when fish were exposed to RO retentates (MacLatchy et al. 2001). In studies at other mills, we have successfully used standardized toxicity tests to identify waste streams that have the potential to be associated with endocrine disruption and also to determine sublethal exposure concentrations for endocrine disruption studies (Rickwood et al. 2006).

Initial chemical analysis of the RO feed identified phenols and glycols as the dominate constituents, with guaiacol as the main phenol constituent removed by RO (it was not found in permeate and was concentrated 100-fold in the RO retentate) (Dubé and MacLatchy 2001). Manool-type alcohols from wood extractives were likewise found in the RO feed, not in the permeate, and concentrated in the RO retentate. The RO feed, permeate, and retentate contained little in the way of resin or fatty acids, and sterols were not detected (Dubé and MacLatchy 2001). Weak black liquor (WBL) was shown not to be entrained in the 5th effect condensate (RO feed) stream at IPP (Dubé and MacLatchy 2001). Both chemical characterization and tracking (using conductivity) of WBL entrainment eliminated nonvolatile, high molecular weight compounds usually found in WBL as potential compounds in the condensates responsible for endocrine depression.


It has proven difficult to identify causative compounds in pulp mill final effluent due to the complexity of final effluent and the number of unidentified chemical compounds (Hewitt et al. 2008). Furthermore, the residual lignin content of wood fibres in the tree make up a large component of the effluent matrix, and these large molecules make it extremely difficult to study smaller bioactive substances. The condensate stream is a much less complex waste stream than the final effluent stream and does not contain any lignin. Therefore, we began to focus our efforts on characterizing the constituents of this waste stream because it was linked to endocrine changes in fish. We began by confirming that the bioactivity of the RO feed was found post-RO in the retentate and not with the permeate (MacLatchy et al. 2001). Successes in this second phase were directly linked to the development of chemical extraction techniques and in the refinement of our mummichog bioassay method for endocrine toxicity.

A solid-phase extraction (SPE) method was developed by Hewitt et al. (2002) to isolate chemical recovery condensate extractives for the evaluation of their hormonal activity. In this work, hormone depressions in mummichog were used to direct SPE method development. Ultimately, a two-stage SPE method was developed that recovered compounds causing hormone depressions. In the confirmation experiment, male and female mummichog were exposed (seven-day static exposures with daily renewal) to whole condensates, extracts from suspended particulates (>1 μm), two fractions from the first SPE (SPE-1 [styrene divinylbenzene] ethyl acetate fraction, SPE-1 methanol fraction), and one SPE-2 (reversible graphitized carbon) fraction, and residual condensates after SPE. The SPE-1 methanol fraction, SPE-2 extract, and solids extract showed significant reductions in plasma testosterone while the residual condensates following SPE showed no hormone effects. It was concluded that the SPE methodology completely recovered activity from the whole condensates. Future work focused on the SPE-2 fraction since it exhibited the greatest potential for depressing hormones in both sexes. A comparison study showed that condensates derived from both hardwood and softwood batch production runs at IPP had an equal potential to lower hormone levels (Hewitt et al. 2002). In terms of identifying characteristics of the compounds involved, this evidence showed them to be organic, water soluble, and readily bioavailable to fish. These characteristics also were consistent with previous studies, showing a rapid onset and recovery of steroid responses in wild fish following mill shutdown (Munkittrick et al. 1992).

Since Phase 1, we had been working on the optimization and standardization of short-term exposure bioassays with mummichog, including the validation of effects from model endocrine disruptors (MacLatchy
et al. 2003; Sharpe et al. 2004). In brief, mummichog are collected from clean reference estuaries in New Brunswick and transported to laboratory facilities at the University of New Brunswick in Saint John, where they are acclimated for at least two weeks prior to experimentation. Fish are maintained at a natural photoperiod in 16 ppt salinity seawater and dissolved oxygen (>80%) in a flow-through system. Fish are fed daily with commercial trout pellets and water quality measurements are conducted daily in order to maintain standardized conditions. Subsequent to laboratory acclimation, three or four adult mummichog (minimum 65 mm) of each sex are weighed and randomly allocated to glass aquaria. Fish are held in the experimental aquaria for one week prior to the commencement of treatments; during this acclimation, the fish are maintained in static tanks with carbon filters. Throughout the acclimation period and exposure, fish are kept at a 14-h light to 10-h dark photoperiod (late spring conditions), and fed commercial trout pellets daily. Each tank is aerated and water quality parameters are recorded. Temperature is approximately 16 to 18°C for each exposure. During the exposure period, water is completely renewed and extracts/effluents are re-administered every 24 hours for 7 to 14 days to each tank by netting fish from each tank, placing them momentarily in a bucket, emptying and refilling the tanks, and replacing the fish. Seven-day exposures have been shown to be adequate for eliciting responses in endocrine status in mummichog exposed to pulp mill effluents and model compounds (Hewitt et al. 2002; MacLatchy et al. 2003). Extracts are dissolved in methanol; reference tanks receive equivalent doses of methanol (0.0125% vol/vol) for control purposes.

To the end of 2002, the IOC methodology had progressed from initial identification of a particular waste stream associated with endocrine responses in fish (Phase 1 or Source Identification) to: 1) development of an optimized SPE method that completely recovered chemicals that reduced testosterone levels in mummichog; 2) characterizations of active fractions by gas chromatography-mass spectrometry (GC-MS) which revealed compounds possessing functionalities consistent with lignin degradation products; and 3) development and refinement of a short-term reproductive bioassay for the mummichog to determine biological activity of pulp mill effluent constituents.


Preliminary GC-MS analyses of the most potent extract to affect steroid levels in mummichog (SPE-2) indicated that condensate extractives associated with steroid depressions in mummichog had properties consistent with lignin degradation products (Hewitt et al. 2002). This information provided some insight into the classes of chemicals involved and was designated as an additional tier of information within our causal frameworks (Hewitt et al. 2005). Further work (Belknap et al. 2004, 2006) was undertaken to progress to the next tier and to ascertain more information regarding the chemicals involved in the responses.

The primary objectives of this phase of our studies were to: 1) develop high pressure liquid chromatography (HPLC) methods to fractionate the SPE-2 extract on a preparative scale for testing using mummichog in the seven-day static renewal assay; 2) isolate chemicals responsible for plasma testosterone depression in mummichog; and 3) identify candidate plasma testosterone depressing substances in condensate extracts, in lieu of definitive bioassay findings. As part of method development, we also evaluated the chemical stability, consistency, and composition of the hormonally-active condensate extract over time and under various production conditions at the mill.

**HPLC fractionation and fish exposures.** The preparative HPLC method was first optimized to resolve condensate components detected by ultraviolet light absorption and was determined to be adequate for bioassay testing using the mummichog in vivo seven-day bioassay. The objective of the first experiment was to determine which fractions of the SPE-2 bioactive condensate extract caused significant depressions in plasma testosterone at 1% (vol/vol) condensate equivalents. Exposures consisted of a positive control (total SPE-2), a reference (laboratory blank), and the six HPLC fractions of SPE-2. Responses in fish exposed to the whole extract at 1% vol/vol were not as pronounced as had been observed previously, and activity in only one of the fractions was weak for only one sex. A repeat experiment at 1.5% (vol/vol) also was inconsistent (Shaughnessy et al. 2007).

The next exposure was carried out to determine the responses in plasma testosterone following exposure to 1 and 4% of the RO feed (Shaughnessy et al. 2007). A secondary objective was to determine if potential compound degradation throughout the duration of the seven-day exposure would have an impact on responses observed. Therefore, for both 1 and 4% treatments, RO feed was collected once from the mill and the same stock of condensate was maintained at room temperature and used throughout the exposure (batch treatments). As well, 1 and 4% samples of the RO feed were collected daily and administered to the aquaria such that a new stock of feed was used each day (daily treatments). It was determined that 4% vol/vol was required to elicit significant plasma steroid responses in males, while females did not respond. Significant decreases in circulating testosterone were observed in male mummichog exposed to the 4% RO feed whether collections were made daily or only once at the beginning of the exposure, demonstrating consistency and stability between condensates collected daily during a one-week period and those stored for one week (Shaughnessy et al. 2007).

One question remaining from this series of exposures was whether SPE-2 did in fact contain the biological
chemicals of the RO feed capable of depressing hormone levels. Therefore, we exposed mummichog to 0.5, 1, 2, and 4% SPE-2 (reference group received methanol) to compare results to the previous 1 and 4% RO exposures (Shaughnessy et al. 2007). Again, female mummichog did not show any responses in circulating testosterone levels for any treatment. Male fish exposed to 4% SPE-2 showed the greatest depression in plasma testosterone relative to the other treatments when compared with control values. Therefore, it was concluded that the SPE-2 fraction is a good representation of the RO feed since consistent effects were found in both females and males (with males responding in both experiments at the 4% concentration) (Shaughnessy et al. 2007).

Employing these findings, a third experiment (carried out in April 2004) was then conducted using a 4% (vol/vol) condensate equivalent exposure and HPLC fractionation of the SPE-2 extract. As observed previously, no significant responses in plasma testosterone levels were observed in females (Shaughnessy et al. 2007). Plasma testosterone was significantly reduced compared with reference fish in males exposed to the 4% RO feed. However, perplexingly, males showed significant increases in plasma testosterone for two fractions.

Condensate constituents. These results led to questions regarding condensate handling, extraction, and fractionation, as well as the temporal quality of condensates. Subsequent work focused on these areas. Condensates were collected at various time intervals over a six-month period, extracted by SPE, and analyzed by GC-MS. Concentrations of confirmed condensate extractives were consistent in all samples collected. However, spiking experiments of confirmed extractives did reveal substantial losses following HPLC fractionation and three different methods of fraction preparation (Belknap et al. 2006). In an effort to determine candidate chemical classes involved in the hormone depressions, all unique extractives were catalogued by mass spectra, peak area, and retention time. Nine compounds were confirmed and quantified against authentic standards. Confirmed components included a range of phenolic guaiaacyl-based lignin degradation products, sulfur (S8), three diterpenoids, and a dimethoxy pinosylvin stilbene (Belknap et al. 2006). Candidate chemicals associated with steroid depressions were identified following a set of predetermined criteria based on previous observations of steroid disruptions whereby the RO feed and RO retentate depressed testosterone, while the RO permeate had no effect in males, and a 25% (vol/vol) exposure was required to induce a depression in females (Dubé and MacLatchy 2001; MacLatchy et al. 2001).

Out of 39 unique components in bioactive SPE extracts of the condensates, six were associated with hormonal activity. Mass spectral interpretation indicated hydroxylated diterpenoids, sesquiterpenoids, and a lignin-derived stilbene as classes of chemicals associated with steroid depressions (Belknap et al. 2006).

A logical next step in our work at IPP was to determine if the same streams and chemicals were involved in effects on fish reproduction at another mill. We examined 5th effect condensates from a bleached kraft mill in Ontario, Canada that had an established history of effects on wild fish. Semiquantitative concentrations of the six candidate compounds were consistent in extracts of softwood condensates at both mills with the exception of some diterpenoids, suggesting that these classes of compounds may be involved in the responses.

Improvements in condensate handling and chemical identification. The reduced potency of the condensates to alter steroid profiles in fish as compared with previous work (Dubé and MacLatchy 2001; Hewitt et al. 2002) was a significant finding of the exposures in Phase 3 (Shaughnessy et al. 2007). However, these changes could not be linked to mill operations since no known changes in processes have been made since 2000 (D. Muir, Environmental Manager, IPP, personal communication). Our experiments also demonstrated that the fractions derived from current methodologies were highly variable in their effects. Therefore, from 2004 to 2008, the focus was on improving condensate handling, analysis, and fractionation methodologies before any further work was undertaken. Overarching goals were to improve our capacity to handle and characterize condensates at IPP so that we can eventually work toward understanding the extent of similarities and differences among condensates at pulp mills, to resolve whether putative endocrine-active compounds in IPP (or other mill condensates) can be traced through mills to final effluents, and to determine the potential of RO to be applied as a waste stream treatment at other mills.

The first step was to re-evaluate the fractionation methodology in an effort to “pinpoint” where exactly the active compounds were being lost (Milestone et al. 2008). To begin this, two sample handling parameters were evaluated regarding recovery: 1) delay between sampling and extraction, and 2) the evaporation of solvent during sample preparation. We first evaluated changes in the condensate samples over time with respect to the transport of samples from IPP in New Brunswick to our laboratory in Ontario. Changes in the profile of SPE-2 chromatographs were monitored over time (Fig. 1). It was shown that if the condensate sample was refrigerated for twelve days, almost nothing could be detected. Freezing the samples prior to shipment was also considered in an attempt to reduce sample loss prior to extraction. However, comparison of chromatographs between condensate samples that were immediately extracted and those that were initially frozen for seven days showed a large decrease in detected compounds for the frozen samples. This implies that the condensate samples must be shipped and processed as quickly as possible, rather than storing and processing the samples at a later time.
As it had been determined that substantial losses were being observed following HPLC fractionation, this was the next step in sample handling that was considered. Since chromatographs from the HPLC showed many peaks, meaning that the compounds were eluting off the column, it was thought that the losses may be occurring during the solvent evaporation process. The solvent used during the HPLC fraction of SPE-2 was a solvent gradient of acetonitrile and water ranging from 5 to 50% water. An experiment was undertaken where standards confirmed in condensates were dissolved in a range of organic solvents as well as mixtures of acetonitrile and water. Each solvent or solvent mixture was subsequently evaporated under a gentle stream of nitrogen as per normal evaporation procedures. For all compounds dissolved in an organic solvent, including 100% acetonitrile, it was possible to recover 100% of the compounds as detected by GC-MS (Fig. 2). However, for the water/acetonitrile mixtures, lower recoveries were observed, with recoveries decreasing with increasing proportions of water. This showed that a large proportion of the known compounds in condensates were partially volatilized at conditions similar to those required to evaporate water.

The confirmed condensate compounds were also analyzed for recovery after all the solvent had evaporated (Fig. 3). Recoveries of phenolics were significantly reduced minutes after “just dryness.” While it took much longer, diterpenes such as manool and geranyl linalool also showed reduced recovery after the initial solvent had evaporated.
This showed that, firstly, the presence of water (after SPE) will greatly reduce any recovery of compounds, and that secondly, following solvent evaporation, dry samples should not be subjected to further nitrogen evaporation and that care needs to be taken to ensure samples are evaporated to “just dryness.” It is likely that a combination of both of these factors led to the loss of activity following the production of fractions from the HPLC separation of SPE-2 (Shaughnessy et al. 2007).

The methodology for sample analysis was also re-evaluated. The analytical concentrations profiled by GC-MS were increased approximately four-fold, GC oven temperature programming was optimized for enhanced resolution, and run times were extended. This led to the discovery and confirmation for the first time of five plant sterols (cholesterol, campesterol, stigmasterol, stigmastenol, and sitosterol) along with squalene (a terpenoid metabolic precursor to plant sterols) present in condensates. A further five additional sterols were tentatively identified.

Because the previous HPLC fractionation method (Belknap et al. 2006) produced large volume fractions, it became necessary to re-evaluate how the SPE-2 extract was fractionated. A method that was entirely nonaqueous was required to ensure full recovery of compounds extracted from the condensates. Experiments with normal phase chromatography proved unsuccessful, and attempts at eluting the graphitized carbon SPE with mixtures of different solvents of decreasing polarity only showed a washing effect, rather than differences in the polarity of the eluting solvent.

Since the SPE-2 cartridge containing graphitized carbon can occasionally bind organic compounds irreversibly (Hennion 2000), subsequent method development focused on SPE-1 from the original method (Hewitt et al. 2002). The original method eluted compounds from SPE-1 using ethyl acetate followed by methanol. Both of these solvents are considered to be reasonably polar. The resin of the SPE-1 cartridge contains a crosslinked polystyrene divinylbenzene copolymer capable of extracting compounds with a wide range of polarities. Further elution of the SPE-1 cartridge using very nonpolar solvents (toluene followed by hexane) revealed large concentrations of previously unknown material. Comparison of the GC chromatographs indicated that this material was similar to much of the nonphenolic material in the ethyl acetate fraction. As such, at least half of the nonphenolic compounds eluted onto the SPE-1 cartridge were in fact not being eluted using the original method and were subsequently not assessed in the bioassay experiments.

One of the difficulties that has been faced in determining the active compounds in these extracts is that many of the compounds have been found in all of the fractions (such as manool). This, along with the knowledge that a considerable amount of material was not being eluted from the SPE cartridges, has meant that we have had to reassess our SPE fractionation regime. We are now looking to produce a robust method that produces chemically-distinct fractions, meaning that each compound is found in only one fraction. This includes re-evaluating available SPE cartridges and the elution solvent order.

**Future Studies and Industry Relevance**

Within the IOC framework, our investigations of the sources and identities of endocrine-active compounds in pulp mill effluent are following several lines of enquiry. We are investigating the changes in potency of the condensates to determine if these are linked to changes in the effluent quality (changes in mill processes). Ongoing efforts in the laboratory are optimizing exposure bioassays using the mummichog (Bosker et al. 2009a,b). Refinement of the bioassay protocol will lessen within- and among-experiment variability. Our focus also continues on the development of new handling procedures for fractionation and account for what now appears to be the semivolatile nature of bioactive substances (Milestone et al. 2008).

Refinement of chemical fractionation and bioassay techniques (including the use of additional in vitro bioassay techniques that can be applied to the TIE [toxicity identification evaluation] approach) should enable us to identify the hormone-active compounds in the condensates at IPP and establish a mechanistic basis for these disruptions. Once the compounds at IPP are identified, they can be tracked through to the final effluent to determine their contribution to the endocrine
toxicity of the final effluent (as well as the efficacy of RO for removing the compounds from condensates). The potential of RO to improve effluent quality in the industry can best be assessed once similarities between the condensate and final effluent compositions are confirmed at a number of kraft mills in addition to IPP.

Significant industry-wide questions remain. Are the putative endocrine-active compounds at IPP found in condensates at other mills? Do these compounds survive conventional secondary treatment processes at other mills? If they do, is RO an effective and economical treatment at other mills or are there other options? To what extent are the putative endocrine-active compounds in condensates linked to reproductive effects in fish downstream of pulp and paper mills? To continue to answer some of these questions through IOC, it is important that we advance our understanding of how compounds characterized in the effluent mixture and effects of specific mixtures or compounds active in laboratory bioassays link to effects in fish downstream of pulp and paper mills. This work is ongoing in a number of projects, including those described in this volume (two articles submitted from the National IOC Project: Parrott et al. 2010, van den Heuvel et al. 2010).

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References


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Evaluation of Short-Term Fish Reproductive Bioassays for Predicting Effects of a Canadian Bleached Kraft Mill Effluent

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Under the Canadian Environmental Effects Monitoring (EEM) program for pulp and paper effluents, the observation of a national response pattern of decreased gonad size and increased fish condition and liver size has triggered a centralized multiagency investigation of cause (IOC) of reproductive impacts in fishes. The purpose of the component of the IOC study presented here is to compare a number of fish bioassays for determining reproductive and reproductive-endocrine effects of a bleached kraft mill effluent. The bleached kraft mill chosen for this study had demonstrated the national response pattern in previous EEM cycles. The bioassays employed to examine reproduction were fathead minnow (Pimephales promelas) 5- and 21-d, mummichog (Fundulus heteroclitus) 25-d, and zebrafish (Danio rerio) 7-d tests, all of which had egg production as the primary reproductive endpoint. Additional bioassays examining reproductive-endocrine endpoints included a 7-d mummichog test, a 7- and a 21-d threespine stickleback (Gasterosteus aculeatus) test, a rainbow trout (Oncorhynchus mykiss) 7-d test, and in vitro sex steroid receptor and plasma protein binding bioassays. The zebrafish and fathead minnow reproductive tests showed significant suppression of egg production at the 100% effluent concentration. Endocrine data derived from the tests showed that this effluent did not impact steroidogenic endpoints at any concentration. Bioassays showed that this effluent i) was capable of eliciting cytochrome P4501A induction at as low as 10% vol/vol effluent, ii) was weakly androgenic at 10% vol/vol, and iii) showed no evidence of in vivo estrogenicity. These results were consistent with in vitro receptor binding assays showing a highly variable level of androgenic equivalents over six months of effluent testing, with little evidence of estrogenic activity. Bioassay results were consistent in that the overall conclusion was that this effluent has only a weak potential to cause reproductive impairment and would likely not do so at environmentally relevant concentrations. Field studies and a fathead minnow lifecycle study conducted concurrently were in agreement with reproductive bioassay results as white sucker exposed in the receiving environment no longer had significantly reduced gonadal development. Overall, this study provided evidence that the laboratory assays evaluated for various reproductive endpoints have potential application for future IOC work.

Key words: fish, reproduction, egg production, pulp and paper, effluent, recovery

Introduction

In 1992, the regulations pertaining to the discharge of pulp and paper mill effluents were revised in Canada. In addition to stricter control for the discharge of biochemical oxygen demand (BOD), total suspended solids, and acute lethality, the revised regulations included the establishment of the Environmental Effects Monitoring (EEM) program. A component of EEM examines the effects of pulp and paper effluents on wild fishes. The aim of the EEM program is to use the information from the study of wild fish to assess the adequacy of the discharge regulations on a site-specific basis. The first three cycles of the EEM program showed that pulp and paper mill effluents were causing a eutrophication response in the receiving environment as well as a national pattern of metabolic disruption in fishes (Lowell et al. 2005).

Metabolic disruption, a term given to a pattern of reduced gonadal development associated with greater energy storage in liver and body tissue (Gibbons and Munkittrick 1994), has been a longstanding observation in wild fish populations exposed to pulp and paper mill effluents (Munkittrick et al. 1994). Reduced gonad growth has been associated with a reduction in circulating sex steroid hormones (Munkittrick et al. 1994) and reduced in vitro steroid hormone production in white sucker (Catostomus commersoni) (Van Der Kraak et al. 1992). While exposure to androgens (Ellis et al. 2003), estrogens (van den Heuvel and Ellis 2002), and CYP1A- (cytochrome P4501A-) inducing compounds (Hodson et al. 1992; Munkittrick et al. 1994)
that could be linked to gonad size changes in wild fishes. When effects in EEM studies at individual mill sites are evident for two consecutive cycles, the EEM program calls for Investigation of Cause (IOC) and Investigation of Solution (IOS) studies so that the cause(s) of effects can be determined and so effects can be reduced or entirely eliminated. However, protocols for IOC and IOS studies were not readily available and, as such, a multiagency team was assembled to undertake pilot studies as part of a centralized National IOC Project in Canada.

The first activity of the IOC consortium was a review of the international literature in order to determine if reproductive effects observed in wild fish populations could be linked to mill processes, treatment types, or individual compounds (Hewitt et al. 2008). It was concluded that fishes exposed to the effluent of mills of all manufacturing process types can exhibit reproductive impacts. Where improvement or recovery has occurred, a variety of factors have been implicated such as installation or improved biological treatment, reduction of the release of pulping liquors to the effluent stream, and chlorine dioxide substitution for bleaching of kraft pulps. Thus, despite much work conducted to date, international research remains ambiguous with regards to specific causes and solutions. The review further concluded that for future progress in identifying remedial strategies, the selection or development of cost-effective investigative tools was needed.

Subsequently, the focus of the National IOC Project for reproductive impacts on fishes was on the selection and/or development of diagnostic tools that could be used for IOC/IOS work. One of the first objectives of this work was to comprehensively evaluate existing short-term reproductive tests that are practical for identifying causal agents but are also predictive of metabolic disruption in wild fishes (Hewitt et al. 2008). A bleached kraft mill located on the St. Maurice River in La Tuque, Quebec was selected for initial study because it exhibited reduced gonad size in previous studies (Gagnon et al. 1994). The mill selected for this study produced bleached (DNED, DED; D = chlorine dioxide, E = alkaline extraction, N = nitrogen compounds) kraft pulp for about 1,200 t/d of linerboard, paperboard, and foodboard. The products are made from 40% chips (23% softwood chips, 17% hardwood chips) and sawdust/shavings (mainly softwood). The process water usage at the mill was 87,000 m³/d. The mill effluent was treated in an oxygen-activated sludge plant, with a hydraulic retention time of about 9.8 h, prior to discharge into the St. Maurice River, Quebec (approximately 1% [vol/vol] in the river). Between June and December, 2006, final effluent grab samples were taken from the treatment system outfall by mill staff directly into 1,000-L food-grade plastic totes. Transport times ranged from 1 to 3 d and testing was begun immediately upon receipt of the effluent.

Experimental Overview

Tests conducted were classified to be reproductive bioassays where some measure or reproductive output was quantified (e.g., egg production), or were considered to be reproductive-endocrine bioassays where other reproductive parameters were measured (e.g., steroid hormones: see details of all bioassays in Table 1). Reproduction was directly assessed in three species: fathead minnow, mummichog, and zebrafish. In fathead minnow, two time durations were used in order to validate the use of a shorter-term test. The 21-d fathead minnow test is herein defined as medium-term, while the 7-d test is referred to as short-term. Three species were chosen for reproductive endocrine bioassays, rainbow trout (*Oncorhynchus mykiss*), threespine stickleback, and mummichog (Table 1).

All tests were conducted in flow-through systems with the exception of the zebrafish and rainbow trout tests, which were static with daily renewal. Concentrations for bioassays were chosen to be 0 (diluent water), 1, 10, 30, and 100% vol/vol effluent. The exceptions to this were for the zebrafish bioassay where 65% vol/vol effluent was substituted for 100%, and the stickleback bioassay that did not have a 30% concentration. All flow-through and static exposures had between one and four tank turnovers per day. Fish loading rates varied with the bioassay used and ranged from 0.33 to 3.69 g/L/d. Dissolved oxygen was maintained at >75% saturation for all treatments in all bioassays.

Materials and Methods

Mill and Effluent Collection

The mill selected for this study produced bleached (DNED, DED; D = chlorine dioxide, E = alkaline extraction, N = nitrogen compounds) kraft pulp for about 1,200 t/d of linerboard, paperboard, and foodboard. The products are made from 40% chips (23% softwood chips, 17% hardwood chips) and sawdust/shavings (mainly softwood). The process water usage at the mill was 87,000 m³/d. The mill effluent was treated in an oxygen-activated sludge plant, with a hydraulic retention time of about 9.8 h, prior to discharge into the St. Maurice River, Quebec (approximately 1% [vol/vol] in the river). Between June and December, 2006, final effluent grab samples were taken from the treatment system outfall by mill staff directly into 1,000-L food-grade plastic totes. Transport times ranged from 1 to 3 d and testing was begun immediately upon receipt of the effluent.

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Fathead Minnow Tests

Fathead minnows were held in well water at 25°C with a photoperiod of 16-h light and 8-h dark, and were fed three times daily with frozen brine shrimp, commercial trout food, or fish flakes. The mean (and standard deviation [SD]) weights of fathead minnows used during the exposure were 5.2 (0.3) and 2.3 (0.2) g for males and females, respectively. The short-term and medium-term tests were adaptations of a test developed by Ankley et al. (2001) and have been used for previous work with mill effluents (Martel et al. 2004; Kovacs et al. 2007). For the preexposure phase, the fish were distributed in groups of two males and four females into aquaria containing two spawning substrates. The effluent-exposure phase of the tests was initiated by selecting groups of fish that produced 18 or more eggs per female per day and had three or more spawning events over seven days during the preexposure phase. These groups were randomly assigned to one of the four replicates for each of the five treatments.

Fish and tanks were monitored for the number of spawns, and egg production and egg fertilization were also assessed. Hatching success of the eggs was only monitored in the medium-term test. At the end of the tests, each fish was examined for secondary sexual characteristics using criteria described earlier (Martel et al. 2004). The rest of the fish (including head, tail, and internal viscera except for gonads) were homogenized and assayed for testosterone (males and females), estradiol (females), and vitellogenin activity (males) as described previously (Martel et al. 2004). Water quality was measured daily and the range of temperature was 24 to 26°C, and pH ranged from 7.4 to 8.5.

Mummichog Tests

Adult mummichog were collected in May 2006 from Shediac Bay, New Brunswick with a beach seine. The mean (SD) weights of mummichog used in the experiments were 8.7 (0.6) and 10.4 (0.5) g for males and females, respectively. Fish were maintained in holding tanks maintained at 16‰ salinity, 18 to 20°C, >85% oxygen, and a natural photoperiod. Fish were fed standard trout pellets (Corey Aquafeed, Fredericton, N.B.) twice daily to satiation. Effluent concentrations were adjusted to 16‰ salinity during the exposure. In the reproductive-endocrine mummichog test, adult males and females were exposed for 7 d to determine effects on the reproductive endocrine system. The endpoints included plasma testosterone and 11-ketotestosterone in males and plasma testosterone and estradiol in females, and were measured using an increased precision radioimmunoassay (RIA) for analyzing small plasma volumes (20 to 50 μL) (MacLatchy et al. 2005). Water temperature ranged from 17 to 21°C, and pH from 7.0 to 8.0 across all exposure concentrations.

In the reproductive test, males and females were separately exposed to effluent for 18 d and were then combined in aquaria for 7 d to assess reproductive performance. There were twelve control aquaria and six replicates of all effluent concentrations, each containing 3 males and 3 females. A mesh cage was placed within each aquarium, 2 to 3 cm above the bottom, allowing eggs to fall to the bottom but preventing the adults from consuming them. Eggs were collected by dredging the tank with a fine dip net.

After collection, eggs were placed into Petri dishes at a density of 40 eggs per dish and exposure concentrations were replaced manually in each dish once a day until the eggs hatched. Eggs were visually inspected daily for development of cleavage and any unfertilized or dead eggs were removed. Upon hatching, larvae were transferred to 50-mL beakers containing the appropriate exposure concentrations at a density of 10 larvae per beaker. Fish were monitored for yolk sac absorption and were returned to the original test aquaria (containing 5 L of appropriate treatment) when the yolk sac was fully

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**TABLE 1. Summary of reproductive and reproductive-endocrine tests conducted for the IOC study with La Tuque mill effluent**

<table>
<thead>
<tr>
<th>Test organism/bioassay</th>
<th>Date effluent sampled in 2006</th>
<th>Effluent exposure duration</th>
<th>Endpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>REPRODUCTION BIOASSAYS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fathead minnow medium-term</td>
<td>May 30</td>
<td>21 d</td>
<td>Gonad size, egg production, number of spawns, egg fertilization,</td>
</tr>
<tr>
<td></td>
<td>June 13</td>
<td></td>
<td>egg hatching, secondary sexual characteristics, VTG, plasma</td>
</tr>
<tr>
<td></td>
<td>June 16</td>
<td></td>
<td>steroid hormones</td>
</tr>
<tr>
<td>Fathead minnow short-term</td>
<td>September 5</td>
<td>5 d</td>
<td>Gonad size, egg production, number of spawns, egg fertilization</td>
</tr>
<tr>
<td>Mummichog</td>
<td>May 30</td>
<td>18 d&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Egg production, number of spawns, egg fertilization, gonad size,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hatching success, juvenile survival</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>July 3</td>
<td>7 d&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Egg production, number of spawns, expression of STAR, aromatase, 3β-HSD,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>whole body steroids, and VTG</td>
</tr>
<tr>
<td><strong>REPRODUCTIVE ENDOCRINE BIOASSAYS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mummichog</td>
<td>May 30</td>
<td>7 d</td>
<td>Plasma sex steroids</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>June 13</td>
<td>7 d</td>
<td>Whole body VTG</td>
</tr>
<tr>
<td>Threespine stickleback</td>
<td>December</td>
<td>21 d</td>
<td>In vitro steroidogenesis, spiggin mRNA, VTG mRNA</td>
</tr>
</tbody>
</table>

<sup>a</sup> 18-d effluent preexposure.  
<sup>b</sup> 7-d reproductive assessment.
absorbed and swimming had commenced. Larvae were fed brine shrimp (*Artemia* sp. nauplii) twice daily, and fry food once daily (Rolf C. Hagen, Montreal, Que.) ad libitum. Growth was monitored for six weeks by taking average length measurements from 20 fish in each tank.

**Zebrafish Tests**

Adult zebrafish were received from DAP International (Etobicoke, Ont.). Fish were held in A-HAB units (Aquatic Habitats, Apopka, Fla.) at 28°C in an environmental chamber. Fish were maintained in recirculated well water with a 12-h light and 12-h dark photoperiod. The mean (SD) weight of zebrafish used in the experiments was 0.46 (0.11) g. Fish were fed to satiation two to three times per day with a combination of commercial salmon fry formulation (Martin Mills, Elmira Ont.) and frozen blood worms (Oregon Desert Brine Shrimp Co., Lakeview Oreg.).

Sexually mature adult zebrafish were visually sexed and two male and two female zebrafish were housed in individual static breeding containers with 1 L of well water. These vessels consisted of two plastic containers stacked together. The bottom of the top container was replaced with a mesh so that spawned eggs would fall through and be separated from the fish until collection. Each treatment contained six breeding containers that were placed in a random order in large water baths at 28°C and fish were held in experimental conditions for the 7-d preexposure period. Fish were then exposed for 7 days to pulp mill effluent diluted in well water or well water alone. Water quality was monitored daily and temperature ranged from 26 to 29°C and pH ranged from 7.0 to 8.0 across all treatments.

Eggs were removed by siphoning the breeding containers with a Pasteur pipette and counted. At the end of the exposure, fish were overdosed with MS-222 (Sigma, St. Louis Mo.) and weighed. Ovaries from fish in each breeding container were weighed and stored in RNALater (Ambion, Austin Tex.) at 4°C until separation into primary growth, previtellogenic, and vitellogenic carcases were snap frozen in liquid nitrogen and stored at -80°C prior to extraction of steroids. RNA extraction, reverse transcription, and Real-Time PCR (polymerase chain reaction) followed the methods described by Ings and Van Der Kraak (2006). The genes evaluated included steroid acute regulatory protein (StAR), P450-aromatase (P450-arom), and 3β-hydroxysteroid dehydrogenase (3β-HSD).

**Rainbow Trout Tests**

Immature rainbow trout were used in a 7-d test to measure levels of whole body vitellogenin (VTG) and liver 7-ethoxyresorufin-O-deethylase (EROD). The fish were exposed in 15-L containers for 7 d. Temperature ranged from 12 to 13°C and pH ranged from 7.1 to 8.6 across the treatments. At the end of the exposure period, the trout were weighed, homogenized in a phosphate buffer containing gelatin at 4°C, and centrifuged at 3100 g for 10 min. The resulting supernatants were stored at -85°C until analysis with the rainbow trout vitellogenin enzyme immunoassay kit from Biosense Laboratories (Bergen, Norway). All samples were assayed in duplicate. Hepatic EROD was estimated in postmitochondrial supernatant as an indicator of exposure to hydrocarbons in the effluent. EROD activity was determined using a modification of the fluorescence plate-reader technique outlined by van den Heuvel et al. (1995).

**Stickleback Tests**

Tests with stickleback were to assess exposure to androgens or estrogens in vivo, and methods are published elsewhere (Hogan et al. 2008; Wartman et al. 2009). The mean (SD) weights of stickleback used in the experiments were 1.54 (0.56) and 1.27 (0.35) g for females and males, respectively. Endpoints presented herein are in vitro steroidogenesis, posterior kidney spiggin mRNA production in females, liver VTG mRNA abundance in males, and receptor-binding potency of effluent extracts using rainbow trout brain androgen receptor (AR) and rainbow trout liver estrogen receptor (ER) bioassays.

**Effluent Chemistry**

Effluent samples collected for the short- and medium-term fathead minnow reproduction tests were qualitatively characterized by solid phase microextraction (SPME) using a commercially available apparatus (Supelco). Samples (10 mL) of each effluent were transferred to inert Teflon vessels and stirred at a constant rate with Teflon coated micro stir bars. The SPME fibre with a 100-μm polydimethylsiloxane coating was immersed in the effluent sample for 60 min at room temperature (approximately 22°C). The loaded fibres were analyzed by full scan (m/z 50 to 500) gas chromatography/mass spectrometry (GC/MS) using an Agilent 6890 Series GC coupled with a 5973 mass selective detector. The samples were desorbed in the injection port in splitless mode for 1.5 min at 250°C. The chemical components were separated on a 30-m by 0.25-mm (0.25-μm film thickness) DB-5MS capillary column (J&W Scientific) using a helium carrier flow rate of 1.3 mL/min. The oven temperature program began at 50°C for 5 min followed by a 5°C per minute increase to 260°C and a final hold of 13 minutes. Total peak areas were normalized to the lowest value to obtain a relative index of effluent strength.

**Binding Assays for Goldfish AR and Sex Steroid Binding Protein (SSBP)**

During each week from July to December, effluent samples for binding assays were collected in plastic bottles and stored frozen until lyophilization. For each month, two of the weekly samples were randomly selected for
Reproductive Bioassays for the Effects of BKME

Lyophilized residues were weighed and subsequently Soxhlet extracted sequentially for 12 h using dichloromethane followed by methanol. Extracts were rotary-evaporated and reduced to just dryness using a gentle stream of nitrogen and brought up to standardized concentrations of effluent volume equivalents in dimethylsulfoxide for incubations with the goldfish testicular AR and plasma SSBP as described in detail in Hewitt et al. (2000). The results of each binding assay are presented as effluent concentrations of androgen equivalents, obtained by interpolating competitive extract displacements with accompanying testosterone standard curves. Appropriate laboratory blanks were prepared in parallel with effluent samples and did not differ from the dimethylsulfoxide carrier controls.

Statistics

All statistical comparisons were made at the 5% significance level ($p < 0.05$). When necessary, the data were log transformed to meet assumptions of normality and homogeneity. When the data met assumptions of normality and homogeneity, the mean eggs produced per female per day, number of spawns, percentage fertilization, and percentage hatching from fertilized eggs were compared for significant differences by analysis of variance (ANOVA) with the aquarium being the experimental unit of replication. Where the design allowed, parameters were compared for significant differences using an ANOVA model with aquaria as a nested factor within treatments. For gonad weight, a covariate of body weight was added. In cases when the ANOVA indicated a significant effluent-related effect, Dunnett’s test or the Least Significant Difference test was used to identify the specific effluent concentrations that were statistically significantly different from the control.

Results

The mill effluent tested was not acutely lethal at any of the bioassay concentrations tested. Mortalities for females in the 21-d fathead minnow bioassay were 6.25% mortality in control, 1%, and 30% effluent concentrations, and 12.5% in 100% effluent. For males, mortality was 12.5% in the 1 and 100% effluent concentrations. During the spawning portion of the mummichog reproductive test, mortalities were 0% in the 1% effluent concentration, 16% in the 10 and 100% effluent concentrations, and 25% in the control and 30% effluent concentration due to sea lice infestation. In the zebrafish bioassay, only one male mortality was observed and this occurred in the 30% effluent group (4%).

Egg production in fathead minnow exposed to 100% effluent for 21 d was found to be significantly decreased compared with controls (Fig. 1 and 2). The

Fig. 1. Cumulative egg production in A) the fathead minnow medium-term test, B) the fathead minnow short-term test, C) the mummichog reproduction test, and D) the zebrafish reproduction test.
The effluent did not significantly affect the number of spawns or the percentage of egg fertilization (range 63 to 80%) or hatching success (range 56 to 74%). Gonad size in treatments was not significantly different from controls as measured at the end of the exposure. Secondary sex characteristics of fathead minnows were assessed and ratings for overall colouration of males were found to be significantly reduced by exposure to 100% effluent. On a scale of zero to five, control fish were rated as 2.8 ± 0.16 (mean ± the standard error of the mean [SEM]) and fish at 100% effluent were rated as 2.3 ± 0.18. The difference was attributed to lighter banding. In females, the ovipositor length was significantly shorter in the 100% treatment group (control fish: 4.0 ± 0.13 mm; 100% effluent fish: 3.3 ± 0.16 mm). In males, the number of tubercles, dorsal fin spot, and dorsal pad size were unaffected by effluent.

In the short-term fathead minnow exposure conducted in September 2006, effluent concentration did not significantly affect egg production (Fig. 1 and 2). In this test, egg fertilization rates were significantly lower in the 10% (58%) and 100% (68%) effluent as compared with the controls (90%).

Egg production in mummichog showed a different pattern of effects as compared with fathead minnows. There were statistically significant increases in egg production at the 1 and 30% effluent concentrations (Fig. 1). There were no significant changes in gonad size since it covaries with body weight at the end of the exposure. Larvae hatched from those treatments and raised for six weeks in their respective effluent concentrations showed no significant differences in time to hatch (range 11 to 13 d) or survival over this period (range 74 to 100%).

In zebrafish, the pattern of egg production was similar to that in the 21-d fathead minnow bioassay and was reduced in the 65% effluent exposure, but also in the 10% exposure (Fig. 1 and 2). A reduced spawning rate coinciding with the reduction in egg production was also apparent in the 65% effluent tanks; preexposure testing had 71% of tanks containing eggs after spawning, whereas after effluent exposure began, only 33% of tanks, the lowest rate of the test, contained eggs each day in the 65% effluent exposure.

Biochemical indicators of exposure were measured in three species: threespine stickleback, fathead minnow, and rainbow trout (Table 2). Results show that the effluent was a weak inducer of CYP1A, as measured by EROD in both rainbow trout and stickleback, though this latter observation only held for one of the two timepoints. Examination of VTG as measured directly by ELISA in trout and fathead minnow, and by mRNA transcripts in stickleback, consistently showed no significant elevation in response to exposure to this effluent. The only androgen-linked endpoint examined was the expression of spiggin in stickleback. As with the stickleback EROD data, this endpoint was significantly elevated at 21 d but not at 7 d.

Steroidogenesis and related endpoints were examined in a number of the reproductive and reproductive-endocrine bioassays (Table 3). In the fathead minnow 21-d bioassay, there were no differences in whole body sex steroids in males or females. The only difference observed in the mummichog reproductive endocrine bioassay plasma steroids was a significant depression
Reproductive Bioassays for the Effects of BKME

### TABLE 2. Mean (SEM, n) biochemical indicators of exposure to CYP1A inducers, estrogens, and androgens<sup>a,b,c</sup>

<table>
<thead>
<tr>
<th>Species and endpoint</th>
<th>Relative change at effluent concentration (vol/vol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1%</td>
</tr>
<tr>
<td><strong>CYP1A</strong></td>
<td></td>
</tr>
<tr>
<td>Rainbow trout EROD 7d</td>
<td>ND</td>
</tr>
<tr>
<td>Stickleback EROD males 7d</td>
<td>2.18 (0.01, 11)</td>
</tr>
<tr>
<td>Stickleback EROD females 7d</td>
<td>1.01 (0.30, 7)</td>
</tr>
<tr>
<td>Stickleback EROD males 21 d</td>
<td>1.81 (0.22, 11)</td>
</tr>
<tr>
<td>Stickleback EROD females 21 d</td>
<td>1.71 (0.78, 6)</td>
</tr>
<tr>
<td><strong>Estrogen – VTG</strong></td>
<td></td>
</tr>
<tr>
<td>Rainbow trout 7d</td>
<td>1.00 (0.02, 8)</td>
</tr>
<tr>
<td>Fathead minnow 21 d</td>
<td>0.34 (0.13, 8)</td>
</tr>
<tr>
<td>Stickleback 7 d</td>
<td>0.91 (0.17, 8)</td>
</tr>
<tr>
<td>Stickleback 21 d</td>
<td>0.76 (0.27, 8)</td>
</tr>
<tr>
<td><strong>Androgen – Spiggin</strong></td>
<td></td>
</tr>
<tr>
<td>Stickleback 7 d</td>
<td>1.50 (0.35, 7)</td>
</tr>
<tr>
<td>Stickleback 21 d</td>
<td>4.02 (1.73, 8)</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND = no data.
<sup>b</sup> All endpoints are represented as fold-change relative to the control value (0% = 1.00).
<sup>c</sup> Asterisks (*) indicate significant differences with regard to the control treatment values.

in 11-ketotestosterone in males exposed to 1% effluent. In stickleback no significant exposure-related differences were found in in vitro steroid production measured by testosterone production in gonads from males and females, in estradiol production in ovaries, or in 11-ketotestosterone production in testes (data not shown, see Wartman et al. 2009). In zebrafish, there were no significant differences in in vitro sex steroid hormone production in females and mRNA transcripts of three key genes for steroidogenesis: StAR, 3β-HSD, and P450-arom were not influenced by the effluent exposure.

Lyophilized effluent extracts showed that AR and SSBP binding activity were predominantly in the dichloromethane extract. As levels in the methanol extract were on average only 6% of what was found, the first sequential extraction for both AR and SSBP bioassays, only the dichloromethane extract data is presented (Fig. 3). Levels of testosterone equivalents as measured by AR binding and SSBP varied substantially over the sampling period and ranged between 75 and 600 ng/L. AR and ER binding were assessed independently on the single sample of effluent that was used for the stickleback bioassays. Rainbow trout brain AR binding for a variety of chemical extraction techniques ranged from 189 to 276 ng/L as testosterone equivalent concentrations, while rainbow trout liver estrogen receptor binding ranged from 53 to 93 ng/L as estradiol equivalent concentrations. SPME chemical profile ratios for the four sampling periods (normalized to the lowest total peak area) illustrate the variability in effluent strength since these were 3.3, 3.1, 3.5, and 1.0 for the May 30, June 16, July 13, and September 5, 2006 effluent samples, respectively.

### TABLE 3. Mean (SEM, n) in vivo and in vitro testosterone (T), estradiol (E2), and 11-ketotestosterone (11-KT) production and expression of mRNA related to steroid hormone production<sup>a,b</sup>

<table>
<thead>
<tr>
<th>Species and endpoint</th>
<th>Effluent concentration (vol/vol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
</tr>
<tr>
<td><strong>Whole body steroids</strong></td>
<td></td>
</tr>
<tr>
<td>Fathead T in males (ng/g)</td>
<td>1.77 (0.34, 8)</td>
</tr>
<tr>
<td>Fathead T in females (ng/g)</td>
<td>1.21 (0.17, 8)</td>
</tr>
<tr>
<td>Fathead E2 in females (ng/g)</td>
<td>4.32 (0.69, 8)</td>
</tr>
<tr>
<td><strong>Plasma steroids</strong></td>
<td></td>
</tr>
<tr>
<td>Mumichog T in males (ng/mL)</td>
<td>0.70 (0.29, 12)</td>
</tr>
<tr>
<td>Mumichog 11-KT in males (ng/mL)</td>
<td>1.51 (0.21, 12)</td>
</tr>
<tr>
<td>Mumichog T in females (ng/mL)</td>
<td>0.71 (0.16, 11)</td>
</tr>
<tr>
<td>Mumichog E2 in females (ng/mL)</td>
<td>3.44 (0.55, 11)</td>
</tr>
<tr>
<td><strong>Steroidogenic gene expression and in vitro steroid production</strong></td>
<td></td>
</tr>
<tr>
<td>Zebrafish T in females (ng/pN tissue)</td>
<td>3.51 (0.76, 6)</td>
</tr>
<tr>
<td>Zebrafish E2 in females (ng/pN tissue)</td>
<td>6.36 (0.71, 6)</td>
</tr>
<tr>
<td>Zebrafish StAR</td>
<td>1.00 (0.32, 9)</td>
</tr>
<tr>
<td>Zebrafish 3β-HSD</td>
<td>1.00 (0.39, 9)</td>
</tr>
<tr>
<td>Zebrafish P450-arom</td>
<td>1.00 (0.35, 9)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Zebrafish mRNA endpoints were measured at 65% effluent in place of 100%, are normalized to β-actin, and are represented as fold-change relative to the control value (0% = 1.00).
<sup>b</sup> Asterisk (*) indicates significant difference with regard to the control treatment value.
Discussion

This study demonstrated that a Canadian bleached kraft mill effluent previously associated with reproductive effects in wild fishes had little or no impact on reproductive endpoints as measured using a number of laboratory bioassays. Reduction in egg production only occurred with 65% or full strength effluent. The reduction in egg production was not associated with alterations in steroidogenic endpoints measured in four different bioassays. In vitro receptor binding measurements of effluent extracts and induction of the spiggin gene indicated some exposure to androgens. Stickleback and rainbow trout bioassays also indicated exposure to CYP1A-inducing compounds. All of the bioassays employed consistently showed that this effluent had only a weak ability to cause reproductive effects and only at effluent concentrations well above those in the receiving environment.

A key objective of this study was to determine if laboratory short- and medium-term reproductive bioassays could predict effects observed in wild fish studies or in full life cycle studies. Evaluation of white sucker populations in the receiving environment concurrently with these studies showed that the effluent examined did not cause reductions in gonad size in white sucker captured in the receiving environment (Parrott et al. 2010). However, a reduction in circulating testosterone in males was observed in white sucker captured in the receiving environment of this mill. The results presented here in combination with accompanying work (Parrott et al. 2010) demonstrate that the La Tuque effluent at the time tested does not fit with the national response pattern of metabolic disruption (Lowell et al. 2005).

Effects in the effluent receiving environment of this bleached kraft mill have been the subject of scientific scrutiny for several decades. While initial studies of reproductive effects in wild fish were inconclusive (Hodson et al. 1992), subsequent studies showed that white sucker downstream of the mill were older and larger at maturity and female sucker had significantly reduced gonad development as compared with reference locations (Gagnon et al. 1995). These results were associated with reductions in steroid hormones in females (Gagnon et al. 1994). An additional endocrine effect, the inability of yellow perch (Perca flavescens) and northern pike (Esox lucius) to respond to artificial stress due to structural impairment of adrenal cells was also observed at this time (Hontela et al. 1997).

In addition to these studies, the fish of the St. Maurice River were assessed as part of the Canadian EEM program. During the first cycle of EEM, the mill switched to chlorine dioxide bleaching and a biological treatment plant was installed. These and other subsequent improvements led to a 98% decrease in BOD, a 91% decrease in suspended solids, and a 57% reduction in water usage from 1990 to 2002 (Smurfit-Stone 2002). Despite these improvements, reduced gonad size in female white sucker continued to be evidenced during the first three cycles of the EEM program (Lowell et al. 2005). This and accompanying studies (Parrott et al. 2010; Wartman et al. 2009) represent the first evidence that this mill no longer causes reproductive effects in fishes.

The life cycle test with fathead minnow conducted concurrently with the present short-term tests also showed the limited potency of this effluent to cause reproductive effects (Parrott et al. 2010). In that study, significant increases in egg production were seen at 1 and 30% effluent concentrations, and there was no significant decrease in egg production at 100% effluent as was observed in the fathead minnow medium-term test in the present study. The discrepancy between these results and the zebrafish and fathead minnow medium-term bioassay presented here can likely be explained by variability in effluent quality. The shorter bioassays were conducted during a period of normal operation following a mill shutdown in May, and effluent chemical profiling presented herein indicated that the effluent used in the bioassays conducted in June 2006 had a higher extractable organic content. This was confirmed by the short-term fathead minnow bioassay conducted in September when the effluent was lower in extractable organic content as determined by the chemical extractives profile and in which no reproductive effects were observed. The temporal variability in effluent quality was further indicated by significant fluctuations in a number of other chemical endpoints monitored during the lifecycle study (Parrott et al. 2010) and in the levels of testosterone equivalents from receptor binding assays. High variability in effluent quality has been previously demonstrated with long-term monitoring at other mills and is related to the complex nature of the mill process and treatment operations (van den Heuvel et al. 2010). Such variability represents a significant hurdle for conducting any reproductive or IOC studies with pulp and paper effluent. This underscores the need to understand the current status of mill operations before initiating studies, but also points to the need to conduct replicate experiments with effluents – thus short practical bioassays are essential for IOC work. This observation also provides evidence that minimization of perturbations
leading to treatment system disequilibrium will be critical to the elimination of effects. This observation is consistent with our understanding that releases of substances to the treatment system via spills and condensate handling are a primary cause of reproductive dysfunction in exposed biota (Hewitt et al. 2008).

The observations presented herein demonstrate the conflict between selecting bioassays that are relevant to the primary observation in the field—reduced gonad size—while being sufficiently short and practical for the purposes of IOC work. As a gonad size effect was not observed at the La Tuque mill (Parrott et al. 2010), the ability of laboratory bioassays to predict this effect could not be addressed by this component of the IOC studies. No differences in gonad size were observed in the fathead minnow and mummichog short- to medium-term bioassays presented herein. However, it seems unlikely that gonadal growth could ever be a responsive endpoint in such short bioassays. Evidence from New Zealand indicated that gonad growth in rainbow trout could only be impacted when exposure was initiated before, and not during, gonadal recrudescence (van den Heuvel and Ellis 2002) and that it can take more than an entire reproductive cycle to manifest changes in gonad size due to altered energy intake (van den Heuvel et al. 2008). There is also the question of whether gonad growth in fractional spawners such as fathead minnow, mummichog, or zebrafish is representative of gonad growth in a synchronous spawner such as the white sucker. The only bioassay capable of identifying changes to gonad growth was the longer-term fathead minnow life-cycle bioassay that demonstrated elevated gonad size in both males and females exposed to 100% La Tuque mill effluent (Parrott et al. 2010). Given the primacy of the gonad growth endpoint in the EEM mandate that drives IOC, a medium-term gonadal recrudescence bioassay would be more appropriate as an intermediate step in the IOC process.

It can be concluded that wild fish studies and laboratory bioassays were consistent in demonstrating that at the time of testing, this effluent was not capable of eliciting reproductive effects in fishes at environmentally relevant concentrations. The lack of an effluent-related effect in the laboratory bioassays when there was no effect seen in wild fish provides confidence about the utility of the tests selected for study for IOC/IOS work. In previous studies, egg production was identified as one of the most sensitive reproductive responses to pulp mill effluent exposure (Kovacs et al. 1993; Rickwood et al. 2006a), and egg production has been reduced in fishes exposed to a variety of pulp mill effluents (Martel et al. 2004; Rickwood et al. 2006a, 2006b). While some of those studies occurred at mills where reduced gonad size was observed, these studies were not specifically designed to relate the two endpoints. At one of the mills studied, there was longstanding evidence over two decades of reduced gonad size in wild white sucker (Bowron et al. 2009), yet fathead minnow reproductive tests showed a stimulation of egg production at environmentally relevant concentrations (Rickwood et al. 2006a). The gonad size changes in fishes exposed in the receiving environment is a complex endpoint and may not only be responding to the direct effects of toxicants. For example, a recent study has shown carotenoid depletion in the ovaries associated with impaired reproductive steroidogenesis in fish exposed to pulp and paper mill effluent downstream of a New Zealand mill (Landman et al. 2008). As carotenoids are only made by plants, these changes are likely indirect due to an ecosystem shift from autotrophic to heterotrophic production. The mechanism contributing to reduced egg production in laboratory bioassays may be distinct from the presumed endocrine mechanism contributing to reductions in gonadal development as seen in wild fish. Recent studies have shown the potential for effluents to modulate neuroendocrine function (Basu et al. 2009) and thereby contribute to the rapid cessation of spawning often observed in fish laboratory reproductive tests.

While reproduction in fishes was not impacted by environmentally relevant concentrations of the effluent studied, biochemical indicators of exposure provided some clues as to bioactive agents in pulp and paper mill effluent. In early studies, when the mill did not yet have secondary effluent treatment and used molecular chlorine bleaching, white sucker in the St. Maurice River demonstrated elevated liver CYP1A activity as far as 95 km downstream of the mill (Hodson et al. 1992). The results presented here indicate that the current biologically treated La Tuque mill effluent still contains CYP1A-inducing compounds, but the effluent alone is unlikely to cause this effect in the receiving environment where the maximum effluent dilution is in the order of 1%. Reduction or elimination of CYP1A induction in fishes has been previously observed to occur concurrently with recovery of reproductive parameters (van den Heuvel et al. 2010) or rapidly following cessation of exposure (Munkittrick et al. 1992).

Androgens in pulp and paper mill effluents have been a longstanding observation. One of the earliest studies of the endocrine disrupting potential of pulp and paper mill effluent observed masculinization of female mosquitofish (Howell et al. 1980). Since those studies, the presence of androgen endpoints have been observed in Scandinavian, New Zealand, and Canadian pulp and paper mills (Larsson et al. 2000; Ellis et al. 2003; Hewitt et al. 2000, 2005). While studies in the United States have implicated androstenedione and androstadienedione as androgenic factors (Jenkins et al. 2001), these compounds and a suite of androstanediones could not be detected in the La Tuque mill effluent in samples taken over the course of this study (Wartman et al. 2009) or in other Canadian and New Zealand effluents (Bandelj et al. 2006). The significance of these observations, particularly as they relate to effects on fish gonad size, remains unclear at present.

An important requirement of IOC/IOS studies is the availability of cost-effective laboratory tests which can be used to track effluent quality resulting from
Toxicity reduction manipulations. In the context of the EEM program, the requirement is for tests that could be used to develop strategies that would eliminate the national pattern of metabolic disruption, most notably smaller gonads in wild fish. Tests in the laboratory can assess reproductive performance at various levels of biological organization ranging from the biochemical to the whole organism, such as actual egg production. Usually, biochemical or biomarker responses can be elicited in shorter periods of time and with less effluent requirements, and these are desirable traits for IOC/IOS tests. However, it is difficult to conclusively link biochemical alterations observed in laboratory tests to observations on the condition of wild fish populations. Because the effort for remedial action by industry may be substantial, it is absolutely essential to make sure that any leads from IOC/IOS work will have the desired benefits. Hence the need for the extensive comparison of the responses of the available fish reproduction tests with responses from a fish lifecycle test exposed to the same effluent in the laboratory as well as to the condition of fish exposed to the same effluent in the wild. While it was not possible to directly address several aspects of the intended IOC work at the La Tuque mill, the evidence for the short- and medium-term tests utilized in this study is promising.

Conclusions

This study demonstrated that it is possible to make changes in the operating conditions of a kraft mill that result in improved effluent quality that is reflected in the reproductive performance of fish exposed in laboratory tests as well as in the condition of wild fish. The specific changes leading to this improvement remain to be elucidated in future studies. Since the improvements resulted from compliance with new effluent regulatory limits, this demonstrates the success and value of the regulations as well as the EEM program. The ability of a given mill’s effluent to affect fish reproduction has been shown to be inconsistent, and can be influenced by certain operating conditions and mill shutdowns. This can be a complicating factor for IOC/IOS work that needs to be given due consideration in future studies and demonstrates the importance of close contact/cooperation with mill staff. At the same time, the variations in effluent quality resulting from the changes in specific mill operating conditions are providing valuable leads concerning mitigation strategies.

The egg production endpoints evaluated in this study were sufficiently sensitive to track changes in effluent quality, and this is strong confirmation for the utility of such tests in IOC/IOS work. While it was not possible to identify laboratory test(s) best suited for assessing specific effluent-related effects on the gonad size of wild fish, the laboratory tests selected for evaluation in this study all appear to have good potential for IOC/IOS work. Further studies are currently underway with mill effluents affecting gonad size in wild fish, and which will provide the full evaluation necessary for test selection and ultimately for progress on solutions for the pulp and paper sector.

Acknowledgements

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References


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Responses in a Fathead Minnow (*Pimephales promelas*) Lifecycle Test and in Wild White Sucker (*Catostomus commersoni*) Exposed to a Canadian Bleached Kraft Mill Effluent

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To evaluate currently available bioassays for their use in investigating the causes of pulp and paper mill effluent effects on fish reproduction, the responses of wild white sucker (*Catostomus commersoni*) collected from the receiving environment at the bleached kraft mill at La Tuque, Quebec, were compared with responses of fathead minnow (*Pimephales promelas*) exposed to effluent in a laboratory lifecycle test. White sucker collected at effluent exposed sites had increased liver size but none of the reproductive effects that had been documented in earlier field studies at this site. Exposure to 1, 3, 10, 30, and 100% bleached kraft mill effluent (BKME) in the lab led to significantly decreased length, but increased weight and liver size in male fathead minnow. Female length was also decreased and liver size was increased at high effluent exposures. Most effluent concentrations (1 to 30%) significantly increased egg production compared with controls. The fathead minnow lifecycle assay mirrored the effects seen in wild fish captured downstream of the BKME discharge. These results will be used to select short-term fish tests for investigating the causes of and solutions to the effects of mill effluents on fish reproduction.

Key words: fathead minnow, lifecycle, wild fish, BKME, reproduction, liver size

Introduction

The first three cycles of the Canadian Environmental Effects Monitoring (EEM) program showed that pulp/paper mill effluents were causing general nutrient enrichment in the receiving environment as well as metabolic disruption in fish (Lowell et al. 2005). Metabolic disruption describes fish that are fast growing and are storing more energy (fish are fatter and have larger livers) but have allocated less energy towards reproduction (smaller gonads). When effects are identified and confirmed at a site, the EEM program calls for Investigation of Cause (IOC) and Investigation of Solution (IOS) studies so that the effects may be reduced or entirely eliminated (Hewitt et al. 2008). The current study focuses on the selection and/or development of diagnostic laboratory fish bioassays that can be used for IOC/IOS work. These tests would ideally be able to demonstrate the metabolic disruption pattern observed in wild fish and be practical in terms of duration and volumes of effluent required. Since the ability of shorter-term tests to predict metabolic disruption in wild fish is not known, an evaluation of the available tests was deemed necessary before IOC/IOS work could be initiated.

This manuscript and the accompanying paper (van den Heuvel et al. 2010a) describe the work conducted using effluent from a bleached kraft mill at La Tuque, Quebec, Canada. The work reported here includes an assessment of the responses of wild white sucker (*Catostomus commersoni*) collected from the receiving environment at the bleached kraft mill at La Tuque, Quebec, were compared with responses of fathead minnow (*Pimephales promelas*) exposed to effluent in a laboratory lifecycle test. White sucker collected at effluent exposed sites had increased liver size but none of the reproductive effects that had been documented in earlier field studies at this site. Exposure to 1, 3, 10, 30, and 100% bleached kraft mill effluent (BKME) in the lab led to significantly decreased length, but increased weight and liver size in male fathead minnow. Female length was also decreased and liver size was increased at high effluent exposures. Most effluent concentrations (1 to 30%) significantly increased egg production compared with controls. The fathead minnow lifecycle assay mirrored the effects seen in wild fish captured downstream of the BKME discharge. These results will be used to select short-term fish tests for investigating the causes of and solutions to the effects of mill effluents on fish reproduction.

Key words: fathead minnow, lifecycle, wild fish, BKME, reproduction, liver size

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The objectives of the wild fish collections were: (1) to conduct an EEM-like study with fish exposed to an effluent that was being simultaneously evaluated in multiple laboratory bioassays, and (2) to evaluate additional reproductive responses in wild fish that correspond to the endpoints being measured in short-term tests. These included assessment of circulating reproductive steroids, in vitro steroid hormone production, secondary sex characteristics, gonadal histology, hepatic contents of ligands for sex steroid receptors, and hepatic mixed function oxygenase activity. Fish lifecycle studies were conducted with bleached kraft mill effluent (BKME) from the La Tuque mill and compared to the wild fish assessments (this paper), and ultimately to other short-term tests (van den Heuvel et al. 2010a).

**Methods**

**Mill Selected for Study and Project Outline**

The bleached kraft mill in La Tuque, Quebec (“DNED, DED” where D refers to 100% ClO₂ bleaching, N refers to nitrogen bleaching compounds, and E refers to caustic extraction) produces 1,200 T/d of bleached linerboard, paperboard, and foodboard. Feedstocks consist of 40% chips (23% softwood, 17% hardwood) and sawdust/shavings (mainly softwood). Water usage is 87,000 m³/d for process and 17,000 m³/d for cooling (noncontaminated water). Processing effluent is treated in an oxygen-activated sludge plant with a hydraulic retention time of approximately 9.8 h prior to discharge into the St. Maurice River.

**Wild Fish Capture and Sampling**

Site selection for the wild fish studies corresponded to previous studies on the St. Maurice River, including Hodson et al. (1992), Gagnon et al. (1994), and Alliance Environnement (2007). Three sites were sampled for fish: an upstream reference site 22 km above the Beaumont dam, a near-field exposed site downstream of the effluent discharge but upstream of the dam in La Tuque, and a far-field site downstream of the discharge and 2 km downstream of the dam in La Tuque (Alliance Environnement 2007) (Fig. 1). White sucker were collected from the three selected sites from September 12 to 22, 2006, using overnight sets of 8.9-cm and 10.2-cm mesh gill nets. Fishing at the immediate downstream exposed site was difficult and dangerous due to the strong currents created by the narrowing of the river immediately upstream of the dam and water release. This site represented about 750 m of fishable area and contained habitat that differed considerably from the reference and exposed site below the dam.

White sucker were removed from gill nets and sampled according to McMaster et al. (1991). Fish were placed immediately into a live well and transported to shore for sampling. Blood samples were taken from the caudal vessels using a syringe and heparinized vaccurtainer. Blood was held on ice prior to separation of the plasma by centrifugation; plasma was immediately frozen in liquid nitrogen. Each fish was rendered unconscious by concussion, and was measured for fork length (±0.1 cm) and body weight (±0.1 g). The internal organs were removed and the gonads (±0.01 g) and liver (±0.01 g) were weighed. Male fish were rated with respect to the number and distribution of nuptial tubercle expression according to a subjective scale which ranged from 0 (no tubercles) to 6 (tubercles over entire body). Mesenteric fat was graded subjectively on a scale of 1 to 5 (McMaster et al. 1991). A portion of liver tissue was placed in cryovials and frozen in liquid nitrogen for assessment of mixed function oxygenase (MFO) enzymes, measured as ethoxyresorufin-O-deethylase (EROD) activity (Hodson et al. 1996). The remaining liver was pooled by sex and site on hexane-rinsed aluminum foil and immediately frozen (-20°C) for determination of ligands for goldfish testicular androgen receptors (Hewitt et al. 2000).

A subsample of ovarian tissue was taken from 12 female fish and placed in separate vials with incubation media for subsequent determination of in vitro production of steroid hormones (McMaster et al. 1995). A subsample of ovarian tissue was also collected to estimate total fecundity (total number of eggs per fish). Additional samples of testis and ovary were fixed in Davidson’s solution for histological evaluation. Opercula were obtained from all fish for age analysis (McMaster et al. 1991).

**Steroid Measurements.** Circulating levels of testosterone (both sexes), 17β-estradiol (females), and 11-ketotestosterone (males) from the plasma samples were quantified by radioimmunoassay (RIA) (McMaster et al. 1992). RIA was also used to measure the in vitro production of testosterone and 17β-estradiol (McMaster et al. 1995).

**Histological analysis.** Testes and ovaries were processed according to standard histological methods (fixation in Davidson’s solution and embedded in paraffin). Six to twelve thin sections (4- to 5-μm thickness) were placed on glass slides and stained with hematoxylin and eosin. Five random images at 40× magnification were collected from each testis slide and analyzed using Northern Eclipse (v8.0) software. A 391-point grid was stamped onto each image and the cell types under each grid point were scored. Recorded cell types were spermatogonia, spermatocyte, spermatid, and spermatozoa. The relative proportions of each cell type within a fish were calculated. Images of entire cross sections of each ovary were prepared by stitching image fields at 2.5× magnification together using Northern Eclipse. All cell types containing a visible nucleus within these cross sections were scored and reported, and the size of vitellogenic cells were calculated by tracing. Recorded cell types were primary,
Fig. 1. Map of study area for the collection of wild white sucker upstream and downstream of the bleached kraft mill discharge in La Tuque, Quebec.
cortical alveolar, vitellogenic oocytes, and atretic follicles. The relative proportions of each cell type and the size of vitellogenic cells were used for analysis.

**Hepatic EROD activity and content of ligands for sex steroid receptors.** EROD activity was measured using the methods of Hodson et al. (1996). Frozen liver that had been pooled by sex and site were evaluated for ligands for goldfish testicular androgen receptors according to previous methods (Hewitt et al. 2000).

**Fathead Minnow Lifecycle Test**

Effluent (2,000 L) was shipped weekly (July 2006 to January 2007) from the La Tuque mill to Burlington, Ontario. Effluent was stored at 4°C in stock tanks until ready for use. BKME was mixed in a proportional diluter with laboratory water (dechlorinatetd, UV sterilized, charcoal filtered Burlington city water) to deliver 0, 1, 3, 10, 30, and 100% (vol/vol) BKME to four randomized replicate aquaria (12 L, 25 mL/min), which provided three turnovers per day. Four replicates of each BKME concentration were used along with 8 replicates of controls. Fish loading densities were a maximum 0.5 g of fish per litre per day for adults in the breeding phase. Aquaria water quality parameters were measured weekly (Table 1).

The test started July 20, 2006, and day 1 post hatch was July 25, 2006. The test ended January 29 to Feb 1, 2007. Methods for egg hatching and care of growing fish are detailed in Parrott and Blunt (2005) and Parrott and Bennie (2009). Measures of fish weight were taken on 32, 46, 54, 77, 90, 102, and 133 days post hatch (dph). Fish from each aquarium were weighed live as a group, and mean fish weight was calculated. Each aquarium was culled to 20 fish at 32 dph, 15 at 46 dph, and 12 at 54 dph.

Secondary sex characteristics started to develop between days 54 and 77, and about 23% of fish could be distinguished externally as male or female at 77 dph. Three breeding tiles were added to each aquarium to promote maturation and reproductive behaviours. By 90 dph, 55% of fish were mature. Secondary sex characteristics of about 62% of the fish were evident at 102 dph. At 102 DPh, fish were culled to 8 per aquarium, selecting 5 females and 3 males for the breeding phase of the experiment. All eggs were counted, assessed for fertilization, rolled off tiles, and 100 eggs (or less if the batch was <100 eggs) were removed to hatching cups in aerated laboratory water.

At 168 to 171 dph fish were sampled as described in Parrott and Blunt (2005) with the following exceptions. Male secondary sex characteristics were assessed as follows: dorsal fin dot was graded as absent (0 points) or present (1 point); dorsal fat pad was graded on scale of 0 (no pad) to 5 (very well-developed pad); nuptial tubercles were counted under a dissecting microscope, as were the number of large, prominent tubercles; banding was assessed on a scale of 0 (no banding) to 5 (fish with a banding score of 4 had very dark pronounced banding, whereas a score of 5 was for fish with pronounced banding and a dark head; fish with a dark head and no banding received a score of 2); Male Index was calculated as the sum of fin dot score + dorsal fat pad score + (# tubercles + # large tubercles)/5 + banding score. The Male Index ranged from 1.7 to 15.2.

For females, ovipositor length and width were measured under a dissecting microscope, and triangular ovipositor area was calculated as length × width ÷ 2. No females had male external sex characteristics. At the termination of the experiment, there was only one immature fish.

**Statistical Analysis**

Male and female in both tests were analyzed separately. Examination of the potential site or concentration (lifecycle) differences in fish length and body weight were evaluated using analysis of variance (ANOVA). Body weight with length as a covariate (condition factor), gonad weight with body weight as a covariate, liver weight with body weight as a covariate, and number of eggs (fecundity) with length, weight, and gonad weight as covariates were evaluated using ANCOVA (analysis of covariance). Data for fathead minnow were presented as somatic indices such as condition factor (K = weight/length³), gonadosomatic index (GSI), and liver somatic index (LSI). Data were checked for normality and

**TABLE 1. Mean ± standard deviation water quality parameters in fish exposure aquaria from BKME exposures and control water**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature (°C)</th>
<th>Dissolved O₂ (mg/L)</th>
<th>pH</th>
<th>Conductivity (μS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.2 ± 0.46</td>
<td>7.67 ± 0.49</td>
<td>7.75 ± 0.22</td>
<td>345 ± 15</td>
</tr>
<tr>
<td>1% BKME</td>
<td>24.1 ± 0.42</td>
<td>7.72 ± 0.39</td>
<td>7.76 ± 0.16</td>
<td>366 ± 19</td>
</tr>
<tr>
<td>3% BKME</td>
<td>24.2 ± 0.45</td>
<td>7.59 ± 0.48</td>
<td>7.74 ± 0.21</td>
<td>405 ± 38 ***</td>
</tr>
<tr>
<td>10% BKME</td>
<td>24.1 ± 0.51</td>
<td>7.71 ± 0.45</td>
<td>7.76 ± 0.17</td>
<td>417 ± 29 ***</td>
</tr>
<tr>
<td>30% BKME</td>
<td>24.2 ± 0.54</td>
<td>7.42 ± 0.39***</td>
<td>7.83 ± 0.20*</td>
<td>789 ± 120 ***</td>
</tr>
<tr>
<td>100% BKME</td>
<td>24.3 ± 0.47</td>
<td>7.28 ± 0.54***</td>
<td>8.13 ± 0.25***</td>
<td>1,580 ± 260 ***</td>
</tr>
</tbody>
</table>

*Measurements were weekly; n = 60 for BKME and 120 for control aquaria.

*Asterisks show significant differences from water controls as determined by two sample t test (Bonferroni adjusted p values with separate variance), *** p < 0.001, * p = 0.012.*
evaluated for homogeneity using the Levine’s test prior to analysis; logarithmic transformations were used if data did not meet these assumptions. Nonparametric Kruskal-Wallis tests were used to compare circulating steroid, in vitro steroid production, fecundity, tubercle expression, internal fat stores, and age data between field sites. Length-at-age relationships for wild fish were modelled using a von Bertalanffy equation of the following form: \[ L = L_{\text{max}} \times (1 - 0.96 \times e^{-0.46 \times k \times \text{Age}}) \] where \( L_{\text{max}} \) is the length at infinite time, or maximum length, and \( k \) is the growth constant. Growth relationships were compared using the residual sums of squares method of Chen et al. (1992). Fathead minnow ovipositor area (mm²), Male Index, and F1 parameters (% fertilization, % hatch, % fry mortality, % larval deformities) were assessed for differences among treatments using ANOVA. Significant differences from controls were assessed using two-sample t tests (separate variances, Bonferroni’s adjusted probabilities) to determine levels of significance (asterisks, shown in the text, figures, and tables for \( p < 0.05 \)). All data were analyzed using either Systat 11.0 or 12.0 (Systat Software Inc., San José, Calif.).

Measurement of BKME-Related Chemicals in Fish Exposure Water

Chemical measurements were conducted to provide an indication of effluent quality over the course of the six-month lifecycle assay and for comparisons of effluent quality between samples used for shorter term testing (van den Heuvel et al. 2010a). Effluent samples were collected directly from the lifecycle shipping totes on a weekly basis from May 30, 2006 to January 24, 2007. Dissolved inorganic Carbon/dissolved organic Carbon, major ions (Na, K, and Cl), colour, total metals, and nutrients were analyzed according to protocols at Environment Canada’s National Laboratory for Environmental Testing Laboratory in Burlington, Ontario (Environment Canada 1994).

- Resin acids and plant sterols were analyzed separately according to described methods (Kovacs et al. 2007).
- Total suspended solids and carbonaceous biological oxygen demand analyses were conducted by Environment Canada’s Wastewater Technology Centre according to established protocols (Method INW3 - Determination of Biochemical Oxygen Demand in Water, Method 2540D - Total Suspended Solids). Polyphenol analysis followed the Hach Method 8193 for tannin and lignin and was conducted by the Northwest Aquatic Biology Facility of the National Council for Air and Stream Improvement Incorporated in Anacortes, Washington, U.S.A.

Results

Wild Fish Physiological Parameters

Female white sucker collected downstream of the La Tuque dam were significantly longer and heavier than the upstream reference females. Downstream females also had a significantly increased condition factor and were older than reference females (Table 2). Exposed female white sucker immediately upstream of the dam had significantly different growth compared with fish downstream of the dam. There were no differences between either of those sites and the upstream Beaumont reference location. The difference in the growth of sucker

<table>
<thead>
<tr>
<th>Sex</th>
<th>Parameter</th>
<th>Reference (Beaumont)</th>
<th>Downstream mill (near-field)</th>
<th>Downstream dam (far-field)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Length (cm)</td>
<td>44.1 ± 0.6</td>
<td>42.2 ± 0.9</td>
<td>46.3 ± 0.6 *</td>
</tr>
<tr>
<td></td>
<td>Weight (g)</td>
<td>1,210 ± 46</td>
<td>1,140 ± 63</td>
<td>1,460 ± 52 *</td>
</tr>
<tr>
<td></td>
<td>Condition factor</td>
<td>1.40 ± 0.02</td>
<td>1.31 ± 0.04</td>
<td>1.46 ± 0.02 *</td>
</tr>
<tr>
<td></td>
<td>Gonad weight (g)</td>
<td>45.7 ± 1.9</td>
<td>46.6 ± 2.9</td>
<td>62.2 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>Liver weight (g)</td>
<td>11.4 ± 0.54</td>
<td>15.2 ± 0.98 *</td>
<td>20.1 ± 1.2 *</td>
</tr>
<tr>
<td></td>
<td>Fecundity</td>
<td>28.2 ± 1.2</td>
<td>28.1 ± 1.3</td>
<td>27.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Fat Index</td>
<td>2.6 ± 0.3</td>
<td>1.1 ± 0.2 *</td>
<td>1.6 ± 0.2 *</td>
</tr>
<tr>
<td></td>
<td>Age (yrs)</td>
<td>7.3 ± 0.4</td>
<td>9.1 ± 1</td>
<td>12 ± 0.8 *</td>
</tr>
<tr>
<td></td>
<td>Lmax</td>
<td>46.3 ± 1.1</td>
<td>43.9 ± 1.0</td>
<td>47.3 ± 0.5</td>
</tr>
<tr>
<td>Growth constant ( k )</td>
<td>0.45 ± 0.07</td>
<td>0.41 ± 0.07</td>
<td>0.42 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>( n )</td>
<td>20</td>
<td>11</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>Length (cm)</td>
<td>42.0 ± 0.6</td>
<td>39.4 ± 0.6 *</td>
<td>42.4 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>Weight (g)</td>
<td>1,070 ± 42</td>
<td>930 ± 39 *</td>
<td>1,070 ± 42</td>
</tr>
<tr>
<td></td>
<td>Condition factor</td>
<td>1.42 ± 0.02</td>
<td>1.51 ± 0.03</td>
<td>1.48 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Gonad weight (g)</td>
<td>65.8 ± 3.6</td>
<td>59.9 ± 4.0</td>
<td>69.3 ± 2.7 *</td>
</tr>
<tr>
<td></td>
<td>Liver weight (g)</td>
<td>7.96 ± 0.39</td>
<td>8.88 ± 0.62 *</td>
<td>11.7 ± 0.85 *</td>
</tr>
<tr>
<td></td>
<td>Tubercle Index</td>
<td>2.2 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Fat Index</td>
<td>1.5 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Age (yrs)</td>
<td>8.1 ± 0.7</td>
<td>8.6 ± 0.7</td>
<td>10.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Lmax</td>
<td>44.1 ± 0.7</td>
<td>40.4 ± 1.0 *</td>
<td>43.3 ± 0.9</td>
</tr>
<tr>
<td>Growth constant ( k )</td>
<td>0.47 ± 0.06</td>
<td>0.54 ± 0.07 *</td>
<td>0.52 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>( n )</td>
<td>20</td>
<td>16</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

* Values represent the mean ± SE.

* Asterisk (*) signifies significantly different from reference fish (\( p < 0.05 \)).

* Pound (#) signifies significant interaction in the relationship between the ANCOVA variables between sites.
upstream of the dam was primarily due to a reduced maximum length \( (L_{\text{max}}) \).

Ovary development when expressed relative to body weight or fish length demonstrated significant site differences in the slopes of the regressions. Far-field females appear to invest more energy into reproductive development especially in larger (heavier and longer) fish. Although absolute fecundity numbers are higher in far-field females, relative to length, weight, and gonad size, no significant site differences exist (Table 2). Female white sucker collected from both exposed sites had significantly larger livers relative to the reference females (Fig. 2a). Internal fat stores were significantly reduced in females from both exposed sites (Table 2).

Near-field male white sucker were significantly lighter and shorter than the reference and far-field exposed males, but were similar in age. These males had reduced growth compared with both the far-field and the upstream reference males. This was primarily due to a reduced maximum length \( (L_{\text{max}}) \) that was estimated to be 40.4 cm. There were no differences in growth between the upstream reference and the far-field downstream exposed males.

There were no site differences in the condition of the fish or testes development relative to the length or the weight of the fish (Table 2). Male white sucker liver weights demonstrated significant site differences in the relationship between liver weight and body weight. Near-field exposed male fish demonstrated similar slopes but had larger livers relative to the reference males. Far-field males had significantly different slopes with large fish having significantly larger livers (Fig. 2b). There were no site differences in internal fat stores or secondary sexual characteristics in males (Table 2).

Circulating levels of 17β-estradiol and testosterone were similar between females collected from the three sites (Table 3). Although circulating levels of 11-ketotestosterone were also similar between the three sites, male white sucker collected downstream of the effluent discharge had significantly reduced circulating levels of testosterone (Table 3).

Table 3. Circulating levels of testosterone (both sexes), 17β-estradiol (females) and 11-ketotestosterone (11-KT) (males) in plasma and EROD activity in liver collected from white sucker from three sites on the St. Maurice River around La Tuque, Quebec\(^a,b\).

<table>
<thead>
<tr>
<th>Sex</th>
<th>Parameter</th>
<th>Reference (Beaumont)</th>
<th>Downstream mill (near-field)</th>
<th>Downstream Dam (far-field)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>17β-estradiol</td>
<td>558 ± 96</td>
<td>589 ± 120</td>
<td>621 ± 150</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>273 ± 45</td>
<td>267 ± 62</td>
<td>226 ± 55</td>
</tr>
<tr>
<td>Male</td>
<td>11-KT</td>
<td>1,760 ± 380</td>
<td>2,200 ± 530</td>
<td>1,380 ± 220</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>482 ± 78</td>
<td>289 ± 68*</td>
<td>304 ± 89*</td>
</tr>
<tr>
<td>Female</td>
<td>EROD</td>
<td>0.669 ± 0.047</td>
<td>0.560 ± 0.13</td>
<td>0.599 ± 0.054</td>
</tr>
<tr>
<td>Male</td>
<td>EROD</td>
<td>0.828 ± 0.058</td>
<td>2.11 ± 0.35*</td>
<td>0.961 ± 0.18</td>
</tr>
</tbody>
</table>

\(^a\) Values represent the mean steroid level ± SE.

\(^b\) Asterisks (*) show significant differences compared with reference fish.

\(^c\) Units: 17β-estradiol, testosterone, 11-KT = pg/mL; EROD = pmol/mg protein/min.
Basal production of 17β-estradiol was significantly reduced in follicles collected from females downstream of the dam at the far field site, and stimulated production of 17β-estradiol was significantly increased in near-field exposed females. No other site differences in steroid production were found (Table 4).

Testicular tissue was separated into four stages of development: spermatogonia, spermatocytes, spermatids, and spermatozoa, each representing a stage further in testicular development. There was considerable variability between fish within a site, and when compared between sites, no significant site differences were found (Fig. 3a). Female ovarian development was separated into follicles of primary development, cortical alveolar stages, vitellogenic stages, and atretic follicles. Figure 3b shows that the fish were at the early stages of ovarian development since the majority of the follicles were of the primary stage. There was considerable variability between fish within site and no significant site differences existed.

Hepatic EROD Activity and Ligands for Goldfish Testicular Androgen Receptors

Male white sucker collected immediately downstream of the effluent discharge had significantly higher EROD activity relative to the reference males (2.6-fold; Table 3); no other site differences were present. No statistical differences were detected in the content of androgens in liver of white sucker between sexes and sites (Fig. 4).

Lifecycle Tests

There were no differences in hatching of eggs with exposure to pulp mill effluent. Hatching success was over 90% and survival of fish up to 32 dph ranged from 68% in 30% effluent and to 85% in 100% effluent (data not shown).

Fish were weighed at set times to assess progression of growth over time (Fig. 5). Juvenile fish growth was similar in all effluent treatments from 32 to 77 dph. Fish growth at 90 dph showed slightly decreased weights of fish exposed to 30 and 100% effluent. This trend

Table 4. In vitro steroid production by ovarian follicles collected from white sucker from three sites on the St. Maurice River around La Tuque, Quebec \(^{a,b,c}\)

<table>
<thead>
<tr>
<th>Steroid (pg/10 follicles)</th>
<th>Treatment</th>
<th>Reference (Beaumont)</th>
<th>Downstream mill (near-field)</th>
<th>Downstream dam (far-field)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estradiol</td>
<td>Basal</td>
<td>55.9 ± 7.8</td>
<td>83.1 ± 13</td>
<td>28.4 ± 4.1*</td>
</tr>
<tr>
<td></td>
<td>hCG (^d)</td>
<td>91.0 ± 12</td>
<td>169 ± 22*</td>
<td>80.2 ± 13</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Basal</td>
<td>21.3 ± 3.1</td>
<td>26.5 ± 7.4</td>
<td>14.8 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>hCG</td>
<td>47.9 ± 12</td>
<td>79.5 ± 16</td>
<td>36.4 ± 5.1</td>
</tr>
</tbody>
</table>

\(^a\) Production was measured under basal incubation conditions or following stimulation with hCG.

\(^b\) Values represent the mean production ± SE.

\(^c\) Asterisks show significant differences compared with reference fish.

\(^d\) hCG = human chorionic gonadotropin.

Fig. 3. Histological evaluation of the stage of gonadal development for male (A) and female (B) white sucker collected upstream of the bleached kraft mill discharge, immediately downstream of the discharge, and further downstream of the dam at La Tuque, Quebec.
continued and was statistically significant at 102 dph. At 133 dph, fish could be sexed and males and females could be weighed separately. Weight of males was significantly reduced with exposure to 30 and 100% effluent at 133 dph. At the end of the lifecycle (168 to 171 dph), males from the 30 and 100% BKME treatments weighed significantly less than control males. As well, males from 1, 3, 30, and 100% BKME were significantly shorter than control males (Table 5). Female fish from 10 and 100% BKME were also significantly shorter than the control females at the end of the lifecycle. Condition factors were increased in male fish exposed to 3 to 100% BKME, and in females exposed to 30 and 100% BKME.

The LSI in male and female fish was increased by lifecycle exposure to BKME (Table 5). Male fish LSI was increased with exposure to 1% and 10 to 100% BKME.

**TABLE 5.** Mean values ± standard error for growth parameters and secondary sex characteristics of mature fathead minnow females and males exposed to 1 to 100% BKME or controls (0) for a lifecycle (168 dph).

<table>
<thead>
<tr>
<th>FEMALES</th>
<th>Concentration (%)</th>
<th>n</th>
<th>Length (mm)</th>
<th>Weight (g)</th>
<th>Condition factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>34</td>
<td>0</td>
<td>47.8 ± 0.58</td>
<td>1.25 ± 0.042</td>
<td>1.14 ± 0.022</td>
</tr>
<tr>
<td>1</td>
<td>18</td>
<td>1</td>
<td>48.8 ± 0.71</td>
<td>1.34 ± 0.061</td>
<td>1.14 ± 0.022</td>
</tr>
<tr>
<td>3</td>
<td>14–15</td>
<td>3</td>
<td>47.0 ± 0.71</td>
<td>1.17 ± 0.049</td>
<td>1.12 ± 0.033</td>
</tr>
<tr>
<td>10</td>
<td>17</td>
<td>10</td>
<td>46.0 ± 0.63*</td>
<td>1.15 ± 0.064</td>
<td>1.17 ± 0.037</td>
</tr>
<tr>
<td>30</td>
<td>19</td>
<td>30</td>
<td>46.9 ± 0.76*</td>
<td>1.31 ± 0.067*</td>
<td>1.26 ± 0.038*</td>
</tr>
<tr>
<td>100</td>
<td>18</td>
<td>100</td>
<td>45.3 ± 0.62*</td>
<td>1.30 ± 0.059*</td>
<td>1.38 ± 0.029*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>n</th>
<th>Ovotestis area (mm²)</th>
<th>GSI</th>
<th>Condition factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td>15.5 ± 0.73</td>
<td>1.42 ± 0.10</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>15.7 ± 0.99</td>
<td>1.37 ± 0.12</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>15.4 ± 0.96</td>
<td>1.30 ± 0.078</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>14.5 ± 1.1</td>
<td>1.28 ± 0.10</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td>17.3 ± 0.91</td>
<td>1.10 ± 0.15</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td>19.9 ± 1.3*</td>
<td>1.29 ± 0.085</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MALES</th>
<th>Concentration (%)</th>
<th>n</th>
<th>Length (mm)</th>
<th>Weight (g)</th>
<th>Condition factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>23–25</td>
<td>0</td>
<td>59.0 ± 0.91</td>
<td>2.72 ± 0.15</td>
<td>1.30 ± 0.029</td>
</tr>
<tr>
<td>1</td>
<td>11–12</td>
<td>1</td>
<td>56.3 ± 0.90*</td>
<td>2.61 ± 0.12</td>
<td>1.43 ± 0.037</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>3</td>
<td>55.4 ± 1.0*</td>
<td>2.59 ± 0.14*</td>
<td>1.52 ± 0.045*</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>10</td>
<td>55.8 ± 1.3</td>
<td>2.60 ± 0.21*</td>
<td>1.47 ± 0.042*</td>
</tr>
<tr>
<td>30</td>
<td>11–12</td>
<td>30</td>
<td>51.9 ± 0.96*</td>
<td>2.05 ± 0.14*</td>
<td>1.46 ± 0.054*</td>
</tr>
<tr>
<td>100</td>
<td>11</td>
<td>100</td>
<td>52.1 ± 0.88*</td>
<td>2.01 ± 0.11*</td>
<td>1.41 ± 0.031*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>n</th>
<th>Ovotestis area (mm²)</th>
<th>GSI</th>
<th>Male Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td>1.30 ± 0.086</td>
<td>8.31 ± 0.44</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>1.21 ± 0.11</td>
<td>10.5 ± 0.77*</td>
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<tr>
<td>3</td>
<td></td>
<td></td>
<td>1.41 ± 0.12</td>
<td>9.83 ± 0.66</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>1.07 ± 0.10</td>
<td>10.2 ± 0.66*</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td>1.56 ± 0.15*</td>
<td>7.92 ± 0.50</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td>1.66 ± 0.17</td>
<td>5.18 ± 0.63*</td>
</tr>
</tbody>
</table>

*Bold values with asterisks [*] were significantly different from controls; * = p < 0.05, ** = p < 0.01 but slopes were not parallel in ANCOVA regression.
BKME Fish Lifecycle & Wild Fish Responses

Female LSI was increased by exposure to 30 and 100% BKME. The GSI was unchanged in fish exposed to BKME, except for 100% BKME, which had increased GSIs in both male and female fish compared with control fish.

All fish were visually sexed externally (except one), and external sexing agreed with internal sexing (based on the presence of ovaries or testes). Male external sex characteristics were increased by exposure to low (1 to 10%) concentrations of BKME, and decreased by exposure to 100% BKME. Ovispositor area was unaffected by exposure to BKME, and ranged from 1.10 to 1.42 mm².

There were no effluent-related differences in time to first spawning. Breeding began at 89 dph in one aquarium from each of 0, 3, and 30% effluent exposure concentrations. Other exposure concentrations started breeding at 90 dph (1%) or 93 dph (10 and 100%).

Total egg production was significantly increased in fish exposed to 1 to 30% BKME (Fig. 6). There was an indication of decreased egg production in two of four replicates of fish exposed to 100% effluent, but when all replicates were pooled, variability in egg production among replicates in the 100% effluent exposure was high, so no significant differences from control egg production were detected.

Mean egg diameter and fertilization success did not differ among control and effluent exposed fish. Mean egg diameter ranged from 1.31 to 1.36 mm, and did not differ with treatments. Mean fertilization rate was over 97% for all groups (data not shown).

Egg hatching rates and deformities in fry were assessed in over 5,000 eggs from each pulp mill effluent concentration. Percentages of uneyed dead eggs and mutant eggs did not differ among treatments. Percent hatch of F1 ranged from 76 to 86% and was not significantly affected by BKME exposure treatment (data not shown). Mean fry mortality was 0.7 to 1.5% (±0.71) (in controls) compared with 6.1 to 6.4% (±2.8) in the 100% BKME treatment.

Percentage of deformities in hatched F1 larvae were not significantly increased in a dose-responsive manner with BKME exposure. There were increased deformities in 10% BKME (with 12% of fry deformed). Other treatments and controls had 1.9 to 3.3% deformed fry.

Temporal Concentrations of BKME-Related Chemicals

The majority of effluent chemical parameters showed little fluctuations throughout the sampling period of the lifecycle bioassay. A total of 25 metals were monitored weekly, the majority of which also did not vary greatly during the course of the lifecycle test (combined mean concentration: 41.6 ± 21.7 µg/L), with three notable exceptions (Table 6). There were dramatic increases in aluminum, manganese, and barium (8-, 32-, and 292-fold, respectively) in June relative to the first month of the study (Fig. 7). These high concentrations were generally maintained for the rest of the study until the last week of January 2007 (Fig. 7). These increases occurred in parallel with changes in the qualitative profiles of effluent organics used to obtain a measure of effluent strength (van den Heuvel et al. 2010a).

The total levels of sterols measured were consistently <1.5 µg/L, with the exception of the weeks of the 14th (13.4 µg/L) and 21st (4.5 µg/L) of November (Fig.

**TABLE 6. Summary of physical and chemical characteristics of La Tuque final effluent during the course of the fathead minnow lifecycle study**

<table>
<thead>
<tr>
<th>Effluent characteristic</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOD</td>
<td>6.6 ± 2.4</td>
</tr>
<tr>
<td>TSS</td>
<td>9.3 ± 5.8</td>
</tr>
<tr>
<td>DOC</td>
<td>76.9 ± 19</td>
</tr>
<tr>
<td>DIC</td>
<td>71.7 ± 22</td>
</tr>
<tr>
<td>Polyphenols</td>
<td>25.9 ± 10.0</td>
</tr>
<tr>
<td>Conductivity</td>
<td>1460 ± 310</td>
</tr>
<tr>
<td>Na</td>
<td>264 ± 100</td>
</tr>
<tr>
<td>K</td>
<td>11.1 ± 4.1</td>
</tr>
<tr>
<td>Cl</td>
<td>136 ± 19</td>
</tr>
<tr>
<td>SO₄</td>
<td>221 ± 110</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>304 ± 81</td>
</tr>
<tr>
<td>Colour</td>
<td>368 ± 74</td>
</tr>
</tbody>
</table>

* BOD = biological oxygen demand; TSS = total suspended solids; DOC = dissolved organic carbon; DIC = dissolved inorganic carbon.
* Mean ± SD; n = 32 weeks; units mg/L except where otherwise noted.
* Conductivity units =μS/cm.
Over the course of the study the proportions of sterols making up the totals were relatively consistent, with stigmastanol comprising about 75% of the totals released; the remaining quarter was comprised equally of campesterol, stigmasterol, and sitosterol.

A similar profile in total resin acids was also observed with a peak in concentrations during November 2006 (Fig. 8). The November maximum was 57 μg/L, however additional spikes were seen during the week of June 20th (32 μg/L) and December 7th (23 μg/L). The balance of total concentrations was usually <10 μg/L. The proportions of individual resin acids making up the totals were relatively consistent, with average compositions of 40% attributed to dehydroabietic acid, 20% to abietic acid, 14% to isopimaric acid, and 9% to pimaric acid. The remaining measured acids were <5%. An almost identical pattern in polyphenol content was also observed (data not shown). A November maximum of approximately 60 mg/L was the lone spike in concentrations and contributed to the variability observed in the average (Table 6).

Polyphenol levels were consistent throughout the lifecycle testing, measuring 24.8 ± 8.2 mg/L. However, a sudden and sole peak concentration of 59 mg/L on the November 14th sample provided further evidence that effluent on that week was irregular (data not shown).

Discussion

In evaluating both white sucker collected from the St. Maurice River and fathead minnows in a laboratory lifecycle test, it was determined that the effluent from the La Tuque mill no longer caused metabolic disruption and had limited effects on various measures of reproduction. This represents the first time that the results of an EEM adult fish survey were temporally reproduced in the laboratory.

The mill at La Tuque was selected primarily because prior research demonstrated reproductive effects of the effluent including growth changes, smaller gonads, and induction of MFO activity (Hodson et al. 1992; Gagnon et al. 1994, 1995; Alliance Environnement 2007). Previous work showed that white sucker collected below the dam were older and larger at maturity, and female sucker had significantly reduced gonad development as compared with reference locations (Gagnon et al. 1995). These results were associated with reductions in circulating steroid hormones in female fish (Gagnon et al. 1994). Reduced gonad size in male white sucker continued to be evident in Cycles 2 and 3 of the EEM program (Alliance Environnement 2007). These differences exceeded the EEM critical effect size of 25%, making this mill a candidate for inclusion in the IOC studies for gonad effects.

In contrast, the present study determined no negative effects on gonad size in either wild fish or the fathead minnow lifecycle. Surprisingly, female white sucker from the far-field site had increased gonad size in larger, older fish, and fathead minnows exposed to higher concentrations of effluent also demonstrated increased gonad size. These results correspond well with the mill’s Cycle 4 EEM studies, which were made available following our own field collections (Alliance Environnement 2007).

Upstream reference fish were physically separated from both downstream sites by an upstream dam at Beaumont. Although fish in the near-field zone were collected in the effluent plume, nothing physically prevented them from swimming upstream into unexposed waters for unknown periods of time (Fig. 1). The location of capture at this site was also significantly different in terms of habitat, as very little fishable habitat was available since the river narrowed just before the dam at La Tuque. For this reason, we must interpret site differences in this near-field zone with caution because effects could also be due to the differences in habitat. Far-field fish were isolated from the near-field area by the La Tuque dam, so were exposed to effluent at all times, and habitat here was similar to the reference area.
The metabolic disruption response pattern is a reflection of a change in the ability of fish to allocate energy, resulting in conflicting interpretation of energy storage and energy expenditure indicators. The national response pattern demonstrated in EEM Cycles 2 and 3 was that fish grew faster, were fatter, and had larger livers, but put less energy into reproductive growth. There are components of this response pattern seen at the la Tuque site. Near-field male white sucker were similar in age and demonstrated reductions in growth and increased liver size. This response pattern has been suggested as a transitional progression between a normal population and the metabolic disruption response pattern demonstrated nationally (Munkittrick et al. 2000).

The differences in growth in wild fish could be due to differences in habitats at the reference and downstream near-field sites. They may also be caused by effluent exposure, now or in the past. White sucker were on average 9 to 8 years old so growth changes may have occurred early in life. Far-field female white sucker were older, put similar or greater energy into reproductive growth, and in terms of energy storage, had increased condition and liver weight but reduced amounts of visceral lipid stores. Far-field male white sucker showed no site differences in average age, similar growth and investment in reproduction, and similar condition, but had increased liver size. Collectively, these results show one consistent response: increased liver size. This corresponds to the pattern of liver size in exposed fish from the National assessments of Cycle 2 and 3 data (Lowell et al. 2003). Although larger livers are commonly interpreted as evidence of activated detoxification systems, Lowell et al. (2003) found that liver size paralleled condition and was also similar to weight-at-age relationships indicating that it functions as an indicator of food utilization or storage. Examination of EROD activity did not support increased detoxification contributing to the larger liver size since only male fish at the near-field exposure zone had increased EROD (2-fold) activity. This was also observed in laboratory studies conducted concurrently (Wartman et al. 2009; van den Heuvel et al. 2010a). However, previous detoxification induction data also supports a transition to improved effluent quality over time at La Tuque. Hodson et al. (1992) demonstrated 10-fold induction in fish collected from our far-field location, and 5-fold induction 95 km downstream of the mill’s discharge prior to the installation of secondary treatment and elimination of elemental chlorine bleaching. Both process changes are known to reduce the inducing potential of mill effluents (Bowron et al. 2009). Reduction or elimination of MFO induction in fishes has been previously observed to occur concurrently with recovery of reproductive parameters following cessation of exposure (Munkittrick et al. 1992; van den Heuvel et al. 2010b).

The low levels of EROD induction were also consistent with no detectable differences in the levels of hepatic ligands for goldfish testicular androgen receptors, previously demonstrated in wild fish exhibiting metabolic disruption (Hewitt et al. 2000, 2005). Since accumulated androgens were one of the most consistent responses measured, they were included as an endpoint which could be used to direct IOC work. This study showed that the La Tuque mill effluent contained relatively low and variable concentrations of androgens (maximum 400 ng/L; van den Heuvel et al. 2010a) compared with the previous studies (1,800 ng/L; Hewitt et al. 2005). An overall weak ability of present-day La Tuque mill effluent to affect fish reproduction was associated with no differences in the expression of male secondary sexual characteristics, circulating and in vitro steroid production, or gonadal histology.

Fathead minnows that were exposed for a lifecycle at the same time wild fish were exposed showed a similar response pattern that included increased gonad and liver sizes, and decreased length. Experiments conducted with rainbow trout given controlled rations and exposed to approximately 10% vol/vol of a New Zealand BKME showed reduced growth in a number of experiments, a change in gonad size in some experiments, but no changes in liver size (van den Heuvel et al. 2010b). However, reduced growth was more prominent at higher ration levels and it was suggested that the dark colour of the effluent limited the efficiency of feeding (van den Heuvel et al. 2008). The reduced growth seen in the current exposures was not related to the ability to feed since low effluent concentrations that did not significantly reduce light penetration also lowered growth. Most long-term controlled laboratory studies of pulp mill effluent show increased growth with exposure to bleached sulphite mill effluent (Parrott et al. 2004) and BKME (Borton et al. 2000).

Fathead minnows exposed to La Tuque BKME in the present lifecycle study showed increased (up to 2- to 3.5-fold) egg production compared with control fish. This was unexpected since previous studies with many BKMEs (Robinson 1994; Kovacs et al. 1995; Borton et al. 2000, 2001), several unbleached kraft mill effluents (Borton et al. 2000, 2001), and one bleached sulphite mill effluent (Parrott et al. 2004) reported reductions in the numbers of eggs produced, along with changes in secondary sex characteristics and sex steroid effects. The reduction in growth of the male fish observed in the present study could have been due to increased energy and activity protecting more batches of eggs compared with control fish.

There were no occurrences of male sex characteristics in female fathead minnows, or female sex characteristics in male fathead minnows as was seen previously in pulp mill effluent exposures (Parrott et al. 2004; Rickwood et al. 2006) and with exposure of fathead minnows to ethinyl estradiol (Parrott and Blunt 2005). There was however an increase in male secondary sexual characteristics in male fish at low BKME concentrations (1 to 10% BKME). Similarly, Kovacs et al. (1995) found fathead minnows exposed to ≥2.5% secondary-treated BKME for 275 d had increased male secondary sexual characteristics. In the present study there was also a loss of male secondary sex characteristics in the 100% BKME treatment group.
Similarly, male fathead minnows exposed to 50% of a different Canadian BKME had a delay in appearance of secondary sex characteristics (Robinson 1994). Borton et al. (2000) saw a delay in the development of secondary sex characteristics in fathead minnows exposed to high concentrations of BKME from a mill located in the U.S.A.

Extensive chemical profiling of conventional parameters over eight months illustrated that the La Tuque mill effluent values were within expected ranges for a North American kraft mill biologically treated effluent (LaFleur 1996). Significant fluctuations in some measured parameters were noted, including aluminum, barium, manganese, resin acids, plant sterols, polyphenols, and the levels of ligands for goldfish testicular androgen receptors (van den Heuvel et al. 2010a). These fluctuations are consistent with the qualitative chemical profiling completed at four different times during May to September 2006 (van den Heuvel et al. 2010a). Interestingly, the results initially do not seem to compare since the profiling data indicates a lesser amount of extractable organics while the resin and plant sterol levels increase. In presenting these data to mill personnel, possible explanations for the changes that occurred were related to i) stabilization of effluent quality following a May shutdown that would have contributed to irregularities in June, and ii) a switch in the location where wood feedstocks were obtained in June (G. Desbien, Smurfitstone La Tuque QC, personal communication).

Conclusions

Fathead minnow lifecycle exposures to La Tuque BKME showed decreased growth and increased liver size similar to that observed in wild white sucker captured downstream of the pulp mill discharge. While these findings were unexpected they demonstrate that present-day effluent from this mill no longer causes metabolic disruption. Investigations into what factors are associated with these changes are ongoing. The temporal variability in some of the effluent metals measured indicate fluctuations in effluent quality that must be incorporated into future IOC/IOS studies at selected mill sites. To our knowledge, this is the first study that has compared wild fish metrics of a typical EEM study to a fish lifecycle test run concurrently. The ability of the lifecycle test to reflect the wild fish results demonstrates that the lifecycle test can be used as an anchor for the calibration of more practical short-term laboratory tests for IOC/IOS work targeted towards eliminating effects of mill effluents on fish reproduction.

Acknowledgements

We are grateful for generous funding for this project from the pulp mills in IOC of the EEM. Technical assistance of Beverley R. Blunt, Christine Regan, Gerald Tetreault, Jim Bennett, Chad Boyko, and Ruth Vanderveen is greatly appreciated.

List of Symbols and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>ANCOVA</td>
<td>Analysis of covariance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BKME</td>
<td>Bleached kraft mill effluent</td>
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<tr>
<td>dph</td>
<td>Days post-hatch</td>
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<tr>
<td>EEM</td>
<td>Environmental Effects Monitoring</td>
</tr>
<tr>
<td>EROD</td>
<td>Ethoxyresorufin-O-dethylase</td>
</tr>
<tr>
<td>GSI</td>
<td>Gonadosomatic index</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic O-gondotropin</td>
</tr>
<tr>
<td>IOC</td>
<td>Investigation of Cause</td>
</tr>
<tr>
<td>IOS</td>
<td>Investigation of Solution</td>
</tr>
<tr>
<td>Lmax</td>
<td>Maximum or ultimate length</td>
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<tr>
<td>LSI</td>
<td>Liver somatic index</td>
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<tr>
<td>MFO</td>
<td>Mixed function oxygenase</td>
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<td>RIA</td>
<td>Radioimmunoassay</td>
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References

Alliance Environnement. 2007. Rapport d’interprétation du 4e cycle des ESEE. Smurfit-Stone, Division La Tuque.


Parrott JL, Blunt BR. 2005. Life-cycle exposure of fathead minnows (Pimephales promelas) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. Environ. Toxicol. 20:131–141.


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Effects of Neutral Sulfite Semichemical Pulp Mill Effluent in the Mummichog (Fundulus heteroclitus) Adult Fish Reproductive Test

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Short-term adult fish reproductive tests using freshwater species have demonstrated negative impacts on egg production in fish exposed to complex pulp and paper mill effluents. In an effort to address the ability of laboratory tests to predict effects on wild fish, mummichog (Fundulus heteroclitus) were exposed in saltwater conditions for 21 days to 3 and 30% final effluent of a neutral sulfite semichemical pulp mill that discharges into an estuarine environment in eastern Canada. Although no effects on gonad size, liver size, or condition factor were found, egg production was significantly increased by 31% and decreased by 30% when fish were exposed to 3 and 30% final effluent, respectively. This study i) is the first to demonstrate a decrease in egg production when fish are exposed to complex effluents under estuarine conditions and ii) provides the first linkage of effects on gonad size in wild fish to egg production from laboratory testing in the same species. In so doing, this study also demonstrates the utility of egg production measurements to further investigate the causes and solutions to the effects of mill effluents in both freshwater and marine wild fish.

Key words: Fundulus heteroclitus, endocrine disruption, pulp mill effluent, estuarine environment, short-term adult fish reproductive test, egg production

Introduction

Endocrine disruption has been widely documented in fish exposed to complex effluents, such as municipal sewage (Jobling et al. 2002), pulp and paper mill effluents (Munkittrick et al. 1992), and runoff from agricultural operations (Orlando et al. 2004). There has been special focus on the effect of effluents on the reproductive endocrine system because it is hypothesized that impacts on this system could result in effects on population status (Tyler et al. 1998). A recent whole-lake experiment demonstrated the near collapse of a fathead minnow (Pimephales promelas) population when exposed to environmentally-relevant levels of 17α-ethynylestradiol (EE2), a potent estrogenic compound commonly used in oral contraceptives (Kidd et al. 2007).

Fish exposed to pulp mill effluents in freshwater and marine field conditions, including artificial stream systems, exhibit altered reproductive performance, including reduced gonad size (Munkittrick et al. 1992), delayed maturation (Munkittrick et al. 1992), male-biased sex ratios (Larsson et al. 2000; Örn et al. 2006), masculinized females (Toft et al. 2004), reduced development of secondary sexual characteristics (McMaster et al. 1991), and depressed steroid hormone levels (reviewed in McMaster et al. 2006 and Hewitt et al. 2008).

Laboratory studies are used to study the effect of pulp mill effluent for several reasons: i) as an alternative to field studies due to complex or hazardous receiving environments (Dubé et al. 2002; Bosker et al. 2009a), ii) to investigate the mechanistic background of the effects observed in the field (Van Der Kraak et al. 1992), and iii) as a tool to identify the source of contaminant effects within the mill (Dubé and MacLatchy 2000). The effect of effluents on egg production can be measured in laboratory tests, and can be linked to population-level responses (Miller and Ankley 2004). Studies using fathead minnow exposed to pulp mill effluent (Parrott et al. 2004; Kovacs et al. 2005; Rickwood et al. 2006; van den Heuvel et al. 2010) and Japanese medaka (Oryzias latipes) exposed to sewage effluent (Ma et al. 2005) have been successfully used to identify potential negative impacts of complex effluents on egg production. Exposure of fish to model compounds under marine conditions have demonstrated negative impacts on egg production; exposure of mummichog (Fundulus heteroclitus) to EE2 (Peters et al. 2007) and sheepshead minnow (Cyprinodon variegatus) to 17β-trembolone (Hemmer et al. 2008) resulted in sharp decreases in egg production. The only published short-term reproductive test in which mummichog were exposed to bleached kraft mill effluent under estuarine conditions showed an increase in egg production at low concentrations of final effluent (Melvin et al. 2009). To our knowledge there has never been an adult fish reproductive test done under
estuarine (brackish) conditions that has demonstrated significant reductions in egg production resulting from exposure to complex effluents.

In Cycle 4 (2001 to 2004) of the Canadian Environmental Effects Monitoring (EEM) program, mummichog exposed in the 1% effluent plume of a neutral sulfite semichemical pulp mill located in New Brunswick, Canada had a 44% decrease in male gonad size (AMEC 2007), which is suggestive of the national average response pattern of metabolic disruption observed in wild fish below mills in Cycles 2 and 3 of the EEM Program (Lowell et al. 2005). The goal of the present study was to determine whether effects on egg production could be detected with the adult mummichog reproductive test using a complex effluent with confirmed effects in the field.

**Materials and Methods**

**Fish Holding**

The mummichog is an abundant saltwater minnow found in estuaries along the Atlantic coast of North America from Newfoundland (Canada) to Florida (U.S.A.) (Scott and Crossman 1998). It is used as an environmentally relevant species to study toxicological, genetic, and physiological effects of anthropogenic inputs to saltwater and estuarine environments (Burnett et al. 2007). Short-term tests that measure endocrine and reproductive responses have been developed for mummichog (MacLatchy et al. 2003, 2005; Peters et al. 2007; Bosker et al. 2009a, 2009b). Adult mummichog were collected using minnow traps at a reference site (Saints Rest, Saint John, N.B., Canada; 46°20’N, 64°40’W) in the fall of 2008. Fish were acclimated to laboratory conditions at the University of New Brunswick, Saint John, N.B. Fish were held at 16‰ salinity (filtered Bay of Fundy sea water and dechlorinated City of Saint John water) at ambient temperatures and natural photoperiod in filtered fiberglass circular tanks with dissolved oxygen at >80%. The fish were fed crushed commercial trout pellets (Corey Feed Mills, Fredericton, N.B.) ad libitum every day. Mortalities were minimal in the stock tanks (<5%).

**Mill Description**

The mill selected for this study is located 6.5 km from St. George, N.B., and produces corrugated paper. It utilizes a neutral sulfite semichemical pulping process and uses 90% hardwood and 10% poplar furnishes. In 2008, the mill produced 512 air dry tonnes (ADT)/d of finished medium, using 30% recycled fibres. On a daily basis, about 14,400 m³ of biotreated final mill effluent is discharged into the L’Etang estuary which drains into the Bay of Fundy. The effluent is treated in a primary clarifier, followed by an equalization basin, anaerobic reactors, an activated sludge system, and finally a secondary gravity clarifier. Effluent was collected twice a week, transported to the exposure location (approximately 40 minutes from collection location), and stored in open aerated containers until use.

**Fish Exposures**

Fish exposures were carried out in June and July of 2009 and consisted of preexposure and exposure phases. For the preexposure phase, mummichog (three fish per sex per tank) were randomly allocated to 48 20-L aquaria. Fish were exposed to final effluent at two concentrations: 3% (vol/vol; environmentally relevant) and 30% (vol/vol; tenfold environmentally relevant). Flow rates were constant at two tank turnovers per day. Fish were held in 16‰ saline water at 20°C under a summer photoperiod (16 h:8 h light:dark) at >80% dissolved oxygen. Within each aquarium, a mesh screen made from 3.175-mm plastic mesh (Aquatic Ecosystems, Apopka, Fla., U.S.A.) was placed above the bottom to prevent the fish from eating released eggs. For a period of 14 d during preexposure, eggs were collected twice a week and weighed. These data were used for tank selection based on preset selection criteria (Bosker et al. 2009b). At the end of the 14-d preexposure phase, 24 tanks were selected. The a priori power at the start of the experiment was 85% to detect a difference of >25%. Fish were randomly distributed over the following treatments: i) 3% final treated effluent (Final 3%: the assumed environmentally-relevant concentration); ii) 30% final treated effluent (Final 30%); and iii) control water (Control). Each treatment had eight tanks per treatment and three fish per sex per tank.

Salt (Crystal Sea, CS 150BA Bioassay Laboratory Mixture, Baltimore, Md., U.S.A.) was added to the effluent to reach a salinity of 16‰. Experimental fish were placed in clean tanks, and remaining fish were returned to the stock tanks. Fish were exposed for 21 d and flow rates were constant at two turnovers per day (using the same system as previously described). The effluent was delivered through a serial diluter dosing system (Experimental Solutions, Fredericton, N.B.).

To determine the effect of effluent on fish reproduction, eggs were collected twice a week and weighed (excess water was absorbed by using a paper tissue) for a period of 21 d (a total of 6 egg collections per tank over the 21-d exposure period). Egg data are therefore reported as grams of eggs produced per female. In order to determine whether the average wet weight per egg changed over time among treatments, 50 eggs were randomly selected per tank and weighed once a week. Tanks were checked daily for mortalities; egg production per female was adjusted from the day a mortality was observed. Determining egg production by weight (grams of eggs per female) is strongly correlated to egg production by counting (number of eggs per female), with an R² > 0.98 (Bosker, unpublished data). Therefore, egg production measured in weight of eggs per female can be directly compared with the number of eggs per female.
In the week leading up to the new moon, at day 21 of the exposure (day 35 of experiment; July 13, 2009), adult fish were sampled. Fish were anaesthetized using buffered 0.05% tricaine methane sulfonate (Syndel Laboratories, Vancouver, B.C.). Fish were sacrificed by spinal severance, and measured for weight (±0.01 g), total length (±mm), and gonad weight and liver weight (±0.001 g). Calculations were made of gonad weight relative to body weight (expressed as gonadal somatic index [GSI: 100 × gonad weight/body weight]) and liver weight relative to body weight (expressed as liver somatic index [LSI: 100 × liver weight/body weight]), and total body weight relative to body length (expressed as condition factor; [CF: 100 × body weight/standard length³]).

**Effluent Chemistry**

Final mill effluents collected each week for fish exposures were subsampled for chemical assessments to obtain estimates in the variations of effluent quality during the course of the 21-d exposures. Samples were collected in glass bottles directly from effluent shipping totes when they arrived from the mill. Samples for chemical evaluations were then immediately shipped to Burlington, Ontario overnight in coolers packed with ice for processing. Upon reception in Burlington, effluents were immediately divided and subsampled according to their respective analyses. Time from initial effluent sampling to laboratory analyses was not more than 4 d. Dissolved inorganic carbon / dissolved organic carbon, major ions (Na, K, Cl), colour, total metals, and nutrients analyses were conducted according to protocols at Environment Canada’s National Laboratory for Environmental Testing Laboratory (National Laboratory for Environmental Testing 2008). Total suspended solids and carbonaceous biological oxygen demand (BOD) analyses were conducted by Environment Canada’s Wastewater Technology Centre according to established protocols (Method INW3 - Determination of Biochemical Oxygen Demand in Water, Method 2540D - Total Suspended Solids).

**Statistics**

Statistical analyses were conducted using Statistica 9.0 (SPSS, Chicago, Ill. U.S.A.), with α set at 0.05 and all data reported as the mean ± the standard error of mean (SEM). Data were checked for outliers using Dixon’s outlier test. Log10 transformations were applied to all data. Assumptions of normality and homogeneity of variance were checked using Shapiro-Wilk’s W-test and Levene’s test, respectively. When analyses of covariance (ANCOVAs) were used, homogeneity of slopes was checked prior to running the analyses. ANCOVAs were used to analyze gonad and liver weight relative to body weight, and body weight relative to body length across treatments (fish as replicates). Nested analyses of variance (ANOVAs) were used when comparing body length and weight (fish nested in tanks). To determine whether there was an effect of treatment on average egg weight over time, a repeated measures ANOVA was used. To determine whether there was a difference in the cumulative weight of eggs produced per female among treatments, the total cumulative weight of eggs produced per female at day 14 of the preexposure and day 21 of the exposure were analyzed using an ANOVA (tanks as replicates), followed by a Tukey’s HSD test. To determine at which time-point during the exposure the significant differences among the treatments could be detected, cumulative weight of eggs produced per female during the exposure phase was analyzed using a repeated measures ANOVA; if a significant interaction of time and treatment was detected, data was further analyzed by individual ANOVAs per day, followed by a Tukey’s HSD test. Power analyses were done using PS Power and Sample Size Calculations, which are freely available online (Vanderbilt University 2009), with α set at 0.05.

**Results**

During the course of the study, fish mortalities were minimal; there were two female mortalities in both the control and Final 30% treatment, and three male mortalities in the Final 30% treatment. Effluent chemistry data are presented in Table 1. Most parameters measured were within typical values of final mill effluents, however there were some notably high values that included BOD and colour, particularly when compared with kraft mill effluent (Parrott et al. 2010). The relatively high colour measurements would be expected due to the anaerobic/aerobic sequence during effluent biotreatment (Milestone et al. 2004). Of the suite of 26 metals monitored, all had mean concentrations <7 mg/L, with the exception of barium and manganese, the concentrations of which compare closely with a recently completed 6-month study following bleached kraft mill effluent (Parrott et al. 2010).

In female mummichog there were no significant interactions of slopes of gonad size and liver size relative to body weight and condition factor, and no tank effects in any of the endpoints. There were no significant differences for females among treatments in length (nested ANOVA: \( F_{2,64} = 0.24, p = 0.21 \)), weight (nested ANOVA: \( F_{2,64} = 0.18, p = 0.16 \)), gonad weight (ANOVA: \( F_{2,64} = 0.61, p = 0.55 \)), liver weight (ANOVA: \( F_{2,64} = 0.19, p = 0.83 \)), and condition (\( F_{2,64} = 1.34, p = 0.27 \)) (Table 2). In males there were no significant tank effects for any of the treatments. No significant differences were found for males among treatments in length (nested ANOVA: \( F_{2,63} = 0.20, p = 0.81 \)), weight (nested ANOVA: \( F_{2,63} = 0.55, p = 0.41 \)), gonad weight (ANOVA: \( F_{2,65} = 1.22, p = 0.30 \)), and liver weight (ANOVA: \( F_{2,65} = 1.88, p = 0.16 \)) (Table 2). A significant interaction in slopes was found in male condition factor (\( F_{2,65} = 4.48, p = 0.015 \)) (Table 2); therefore, the analyses could not be continued.
There were no significant changes in egg weight over time (repeated measures ANOVA: $F_{2,42} = 2.26, p = 0.12$), among treatments (repeated measures ANOVA: $F_{2,21} = 0.19, p = 0.83$), or among treatments over time (repeated measures ANOVA: $F_{4,42} = 2.07, p = 0.10$) (Table 3). There were no significant differences in egg production in the preexposure phase (ANOVA: $F_{2,21} = 0.49, p = 0.62$) (Fig. 1). There was a significant effect on cumulative egg production at the end of the exposure phase (ANOVA: $F_{2,21} = 0.48, p < 0.001$) (Fig. 1). There were significantly more eggs produced in fish exposed to Final 3% compared with Control (Tukey’s HSD: $p = 0.015$) and Final 30% (Tukey’s HSD: $p < 0.001$) (Fig. 1A, B). Significantly less eggs were produced in Final 30% compared with Control (Tukey’s HSD: $p = 0.003$) (Fig. 1). There was a significant interaction between time and treatment interaction (repeated measures ANOVA: $F_{10,105} = 5.88, p < 0.001$) for cumulative egg production in the exposure phase (Fig. 2); the significant increase of egg production in Final 3% could be detected from day 11 of exposure onwards (ANOVA: $F_{2,21} = 5.87, p = 0.009$), while the significant decrease in egg production in Final 30% relative to the control was statistically significant at day 21 of the exposure (ANOVA, $F_{2,21} = 19.28, p < 0.001$) (Fig. 2).

### Table 1. Summary of physical and chemical characteristics of Lake Oroopia final effluent during the course of the mummichog exposures

<table>
<thead>
<tr>
<th>Effluent characteristic</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOD&lt;sub&gt;5&lt;/sub&gt;</td>
<td>39.0 ± 0.8</td>
</tr>
<tr>
<td>TSS</td>
<td>271 ± 25.4</td>
</tr>
<tr>
<td>DOC</td>
<td>1297 ± 70</td>
</tr>
<tr>
<td>DIC</td>
<td>643 ± 28.9</td>
</tr>
<tr>
<td>Conductivity</td>
<td>6360 ± 248 μS/cm</td>
</tr>
<tr>
<td>Na</td>
<td>1800 ± 95</td>
</tr>
<tr>
<td>K</td>
<td>78.1 ± 6.3</td>
</tr>
<tr>
<td>Cl</td>
<td>46 ± 1.3</td>
</tr>
<tr>
<td>SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>513 ± 36.7</td>
</tr>
<tr>
<td>pH</td>
<td>8.4 ± 0.09</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>32.6 ± 16.7</td>
</tr>
<tr>
<td>Colour</td>
<td>19866 ± 1050 Pt CO</td>
</tr>
<tr>
<td>Barium</td>
<td>7.3 ± 0.5</td>
</tr>
<tr>
<td>Manganese</td>
<td>26.6 ± 0.6</td>
</tr>
</tbody>
</table>

*Shown are data from weekly samples collected from the weeks of June 21, June 28 and July 5, 2009.
*TSS = total suspended solids; BOD<sub>5</sub> = biological oxygen demand after five days; DOC = Dissolved organic carbon; DIC = dissolved inorganic carbon.
*Mean ± SD (n = 3 weeks); units in mg/L unless otherwise noted.

### Table 2. Summary statistics for male and female mummichog exposed for 21 days to control water, 3% final treated effluent, and 30% final treated effluent<sup>a,b,c,d</sup>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Length (mm)</th>
<th>Weight (g)</th>
<th>GSI (%)</th>
<th>LSI (%)</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>22</td>
<td>80.1 ± 0.6</td>
<td>6.0 ± 0.2</td>
<td>12.8 ± 0.8</td>
<td>4.5 ± 0.2</td>
<td>1.16 ± 0.02</td>
</tr>
<tr>
<td>F3%</td>
<td>24</td>
<td>80.2 ± 1.0</td>
<td>5.8 ± 0.2</td>
<td>11.2 ± 0.8</td>
<td>4.4 ± 0.2</td>
<td>1.12 ± 0.02</td>
</tr>
<tr>
<td>F30%</td>
<td>22</td>
<td>80.5 ± 0.8</td>
<td>5.9 ± 0.3</td>
<td>13.9 ± 1.6</td>
<td>4.4 ± 0.2</td>
<td>1.12 ± 0.02</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>24</td>
<td>71.5 ± 0.7</td>
<td>3.8 ± 0.1</td>
<td>2.1 ± 0.2</td>
<td>3.1 ± 0.1</td>
<td>1.03 ± 0.02</td>
</tr>
<tr>
<td>F3%</td>
<td>24</td>
<td>72.2 ± 0.7</td>
<td>4.0 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>1.04 ± 0.02</td>
</tr>
<tr>
<td>F30%</td>
<td>21</td>
<td>71.3 ± 0.6</td>
<td>3.7 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>2.9 ± 0.1</td>
<td>1.02 ± 0.02</td>
</tr>
</tbody>
</table>

*GSI = gonadal somatic index; LSI = liver somatic index; CF = condition factor.
*C = control water; F3% = 3% final treated effluent; F30% = 30% final treated effluent of a sulfite semichemical pulp and paper mill.
*Values are mean (± SEM).
*Asterisk (*) = a significant interaction (p = 0.015) in slopes.

### Table 3. Average egg weight (mg ± SEM) of mummichog during different phases of the exposure, indicating no difference in egg weight due to treatment<sup>a</sup>

<table>
<thead>
<tr>
<th>Week</th>
<th>C</th>
<th>F3%</th>
<th>F30%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>3.97 ± 0.03</td>
<td>4.01 ± 0.06</td>
<td>4.04 ± 0.02</td>
</tr>
<tr>
<td>Week 2</td>
<td>4.01 ± 0.06</td>
<td>4.00 ± 0.06</td>
<td>4.00 ± 0.08</td>
</tr>
<tr>
<td>Week 3</td>
<td>4.01 ± 0.04</td>
<td>3.86 ± 0.05</td>
<td>4.07 ± 0.04</td>
</tr>
</tbody>
</table>

*C = control water; F3% = 3% final treated effluent; F30% = 30% final treated effluent of a sulfite semichemical pulp and paper mill.
Effect of Pulp Mill Effluent on Egg Production

Fig. 1. Effect of final effluent of a sulfitic semichemical pulp mill on A) the cumulative weight of eggs spawned (grams of eggs/female ± SEM), and B) the average weight of eggs produced (grams of eggs/female ± SEM) in mummichog (Fundulus heteroclitus) exposed to control water (C), 3% final effluent (F3) and 30% final effluent (F30) during both the preexposure (14 d) and exposure (21 d) phases. Different letters indicate significant differences within the preexposure and exposure phases (ANOVA followed by a Tukey’s HSD test, p < 0.05).

Fig. 2. Cumulative weight of eggs spawned (in grams per female ± SEM) throughout the exposure phase. A repeated measures ANOVA was performed to determine whether there was a significant interaction between time and treatment; this was followed by separate ANOVAs for each day individually to determine at which time-point significant differences among treatments were detected. Different letters indicate significant differences among treatments within day (ANOVA followed by a Tukey’s HSD test, p < 0.05).

Discussion

This is the first study in which fish exposed to a complex effluent under estuarine conditions has shown a significant decrease in egg production, measured as cumulative weight of eggs produced per female. The observed pattern was bimodal, with increased egg production at the low concentration and decreased egg production at the high concentration. Similar significant patterns have been observed when fish are exposed to model compounds, including fathead minnow (Pawloski et al. 2004) and Japanese medaka (Tilton et al. 2005) exposed to EE2. The bimodal pattern has also been observed in freshwater studies with three different species exposed to Canadian bleached kraft mill effluent (van den Heuvel et al. 2010). This pattern could indicate hormesis, a fundamental dose response pattern in biological research (Calabrese 2008). However, a study design with an increased number of concentrations is needed to confirm this. More research is needed on the implications of this pattern and the effects on fish reproduction after longer-term exposure.

Following 21-d exposures to any of the effluent concentrations, there was an absence of response in gonad size relative to body weight. This endpoint showed a strong response in the EEM Cycle 4 field study (AMEC 2007) and has been observed as a national average response pattern in fish exposed to mill effluents (Lowell et al. 2005). The lack of a decrease in gonad size in tests of this time duration is a common occurrence in adult fish reproductive tests of multiple species. Reductions in egg production without significantly affecting gonad size has been observed in fathead minnow exposed to effluent from a multiprocess mill (Kovacs et al. 2005), in mummichog (Peters et al. 2007) and Chinese rare minnow (Gobiocypris rarus) (Zha et al. 2008) exposed to EE2, and in Java medaka (Oryzias javanicus) exposed to estrone (Imai et al. 2007). Although there are studies in which both endpoints are affected (e.g., Pawloski et al. 2004; Ma et al. 2005), meta-analyses are needed to determine whether egg production is a more sensitive endpoint than reductions in gonad size. This study did demonstrate, for the first time, that a mill effluent capable of affecting gonad size in wild mummichog also affects egg production in an adult mummichog reproduction test. Whether the two effects are more than associative is not clear at present. However, egg production appears to be a sensitive indicator of the potential of mill effluents to affect fish reproduction (van den Heuvel et al. 2010).
The decrease in egg production in Final 30% could be detected on the last day of exposure (Fig. 2). This confirms a previous study in which it was demonstrated that variation in egg production was highest in the initial 14 days of exposure, and that a duration of 14 to 28 days is needed to minimize variance and optimize the power to detect differences of >25% (Bosker et al. 2009b). A study on bleached kraft mill effluent using mummichog showed an increase in egg production after 7 d of exposure (Melvin et al. 2009). Finding an optimal duration for exposures depends on the sensitivity of the species and/or the endpoint used as well as the potency of the effluent. A shortened fathead minnow test has been developed and successfully applied to pulp mill effluents when the effluent being studied is potent (Kovacs et al. 2007). For initial screening of effluents, an experimental setup with high replication and long duration is most effective to detect more subtle differences in effluent quality (Bosker et al. 2009b); if the effluent proves to be potent, a shortened experimental setup can be used in subsequent studies.

In conclusion, this study is the first to describe a significant decrease in egg production when fish are exposed to complex effluents under estuarine conditions. It is also the first study to demonstrate that egg production in a short-term laboratory test is reduced when wild fish of the same species exhibit reduced gonad size. This study further strengthens the potential to use the mummichog short-term adult reproductive test to test complex effluents under environmentally-relevant estuarine conditions. Future research will be focused on investigation of cause studies (Hewitt et al. 2003) at this mill to determine the source of the reproductive-active contaminants.

Acknowledgements

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Melvin SD, Munkittrick KR, Bosker T, MacLatchy DL. 2009. Detectable effect size and bioassay power of mummichog (Fundulus heteroclitus) and fathead minnow (Pimephales promelas) adult reproductive tests. Environ. Toxicol. Chem. 28:2416–2425.


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Guidance for Site-Specifically Assessing the Health of Fish Populations with Emphasis on Canada’s Environmental Effects Monitoring Program

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Techniques have been developed over the past two decades to site-specifically assess effects of contaminants on the health of fish populations using a sentinel species approach. National environment effects monitoring (EEM) programs have been implemented in Canada for pulp and paper effluents since 1992 and liquid metal mining effluents since 2002 to monitor effects of these discharges on the health of fish populations. The major criticisms of past EEM fish population surveys can be separated into concerns about the adequacy of the reference sites, the potential impacts of confounding factors, the ecological relevance of endpoints used, the influences of natural variability, concerns over statistical design issues, and potential genetic influences on species characteristics. This paper provides input to deal with these issues and guidance on the selection of sentinel species, timing of sampling, and nonlethal sampling methods to evaluate the health of fish populations. Sample size requirements, effect sizes, and power analysis are also discussed as well as data analysis guidance needed to obtain reliable results.

Key words: impact assessment, industrial effluents, sentinel fish species, fish populations, environmental effects monitoring

Introduction

Environmental monitoring frameworks to assess the impacts of contaminants on fish populations have been developed and improved over several years (Colby 1984; Ryder and Edwards 1985; Munkittrick and Dixon 1989a, 1989b; Munkittrick 1992; Gibbons and Munkittrick 1994; Power 1997; Munkittrick et al. 2000; Sandström et al. 2005). These frameworks evaluate environmental impacts on fish populations by assessing changes in population characteristics such as age structure, energy expenditure, and energy storage relative to reference site(s). The goal of the analysis is not to provide a definitive assessment of impacts or causes of impacts, but rather to document changes from reference conditions over time and determine the focus of follow-up studies. This iterative monitoring framework was used in the development of the adult fish population survey component of Canada’s national environmental effects monitoring (EEM) program for industrial wastewaters (Ribey et al. 2002).

The EEM program is a mandatory, regulated, cyclical monitoring program for the pulp and paper and metal mining industries to assess whether mills and mines in compliance with their effluent discharge regulations are associated with environmental impacts on fish or benthic invertebrates (Walker et al. 2002). The pulp and paper and the liquid metal mining effluent EEM programs are currently in their fifth and second cycles of monitoring, respectively, and more than 300 fish population surveys have been conducted as part of the programs. The fish population survey provides an assessment of whether there are differences in the growth, reproduction, condition, and survival of the fish populations between exposed and reference areas or within an exposure area where there are gradually decreasing effluent concentrations.

The EEM program was designed in a cyclical nature to determine whether changes are present at a sufficient size such that additional, more detailed studies are needed. The design of EEM is such that this determination requires confirmation in a subsequent cycle of monitoring, and moving to more detailed monitoring or examination requires that effects exceed a critical effect size (CES). To evaluate the effects of stressors on fish populations, the following questions need to be addressed: Is there an effect? Is the effect stressor related? Is the magnitude and extent of the effect known? Is the stressor-related cause of the effect known?

The major criticisms of past EEM studies (and field studies in general), can be separated into concerns about the adequacy of the reference sites, the potential impacts of confounding factors, the ecological relevance of endpoints used, the influences of natural variability, concerns over statistical design issues, and potential genetic influences on species characteristics. This paper will provide input to deal with these issues and discuss study design considerations, including guidance on the selection of sentinel species, timing of sampling, sample size requirements, nonlethal sampling methods, and data analysis.
Monitoring Level and Endpoints Used

Any “impact” at a biochemical, individual, population, or community level has to be evaluated in terms of the consequences of the change as it affects the sustainability of the ecosystem. Individual organisms may survive numerous biochemical impacts and populations may survive numerous individual impacts. Designing a monitoring program at the fish community level puts constraints on adaptive management (an iterative process of decision making that uses the results of previous studies to provide feedback on whether ecosystem objectives are achieved). Fish community changes are ecologically relevant (but have a long time lag before they are detectable), may be difficult to reverse, and may be the result of changes that are difficult to specifically define. At the other extreme, biochemical responses happen quickly and can more often be related to specific causes, but may have little ecological relevance and be easily reversed. It is important that whatever level is focused on, that the assessment approach balances protection and detection, with reversibility and relevance. The system needs to give enough warning that there is time to respond, but still relates to relevant effects.

Monitoring fish health at the population level has several advantages. The population level approach offers a compromise between the sensitivity and reversibility of biochemical approaches and the relevance of community level endpoints. Monitoring at the community level can potentially miss irreversible, important effects at the population level. Delays in sexual maturity, altered growth, changes in fecundity, and depressions in storage of energy reserves put fish at risk, and knowing this level of risk is important to the management of ecosystems.

In the EEM program, five indicators of fish health or effect endpoints are used to assess fish population health.

These are age (indicator of population survival), weight-at-age and relative gonad weight (indicators of energy use), and relative liver weight and condition (indicators of energy storage). Other data such as fecundity and egg weight are also collected and are used as supporting data. It is important to emphasize that the program is iterative and that the outcome of comparisons is to emphasize areas of focus for subsequent monitoring cycles. For example, impacts showing significant decreases in growth, gonad size, liver size, and condition would emphasize challenges with food limitation and suggest increased emphasis on evaluating food and habitat availability (Table 1) (the EEM program also has components requiring assessment of fish habitat and fish use).

Mean age is meant to give an assessment of the relative ages of the reference and exposed fish populations. If size-selective gear such as gillnets are used, and there is a significant difference in mean ages of fish sampled at both sites with identical gear, the difference points to a need in the subsequent cycles to further investigate the population and the reason for the difference. Methods of aging should be consistent at each sampling area and among cycles, and appropriate quality assurance/quality control procedures followed (e.g., independent confirmation). If fish cannot be aged reliably or if it is not cost/time efficient, the age can be determined by using size-frequency distributions. This may be especially useful when sampling small-bodied fish species or when conducting nonlethal sampling techniques. It may also be possible to confirm the size-frequency distributions by aging representative subsamples from each size class. See Nielsen and Johnson (1983) for more information on size-frequency distributions.

Both size-at-age (growth) and the reproductive measures are meant to give an assessment of the ability of the fish to utilize the food available to them. Growth

<table>
<thead>
<tr>
<th>Age distribution</th>
<th>Energy utilization</th>
<th>Energy storage</th>
<th>Generalized pattern</th>
<th>Cause of changes</th>
<th>Follow-up study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shift to younger</td>
<td>Increased</td>
<td>Increased</td>
<td>Exploitation</td>
<td>Decreased competition between adults associated with mortality or eutrophication</td>
<td>Examine food resource availability and population density</td>
</tr>
<tr>
<td></td>
<td>No change</td>
<td>No change</td>
<td>Recruitment failure</td>
<td>Shift to older age classes associated with decreased reproductive success</td>
<td>Detailed examination of spawning habitat and its utilization, and reproductive development</td>
</tr>
<tr>
<td>Shift to older</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Multiple stressors</td>
<td>Simultaneous impacts on food availability and reproductive success</td>
<td>Detailed studies of reproductive development and food resources</td>
</tr>
<tr>
<td>No change</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Food limitation</td>
<td>Increased competition associated with increased reproductive success or decreased food availability</td>
<td>Examine food resource availability and population density</td>
</tr>
<tr>
<td>No change</td>
<td>Decreased</td>
<td>No change</td>
<td>Niche shift</td>
<td>Modest increase in competition for forage base</td>
<td>Examine food base and competition aspects</td>
</tr>
<tr>
<td>Shift to younger</td>
<td>Mixed</td>
<td>Mixed</td>
<td>Metabolic redistribution</td>
<td>Inability to maximally utilize available food resources</td>
<td>Detailed physiological studies of energetics</td>
</tr>
<tr>
<td>Shift to older</td>
<td>Increased</td>
<td>Increased or decreased</td>
<td>Chronic recruitment failure</td>
<td>Shift to small population of older individuals</td>
<td>Detailed study of reproductive performance</td>
</tr>
<tr>
<td>No change</td>
<td>No change</td>
<td>No change</td>
<td>Null response</td>
<td>No obvious changes</td>
<td>Check population size data to see if carrying capacity of the system has changed</td>
</tr>
</tbody>
</table>

See Nielsen and Johnson (1983) for more information on size-frequency distributions.
is the change in size (weight or length) with time or age. In the case of growth, it may be helpful to collect information on other age classes. For example, it may be important to determine whether there are changes in growth of early life stages. This will assist in determining the magnitude of the effect. Subsequent monitoring should focus on confirming responses detected, and examining the relevance of the changes to other size classes and species.

Reproduction can be expressed as reproductive effort, fecundity, egg weight, or gonad weight relative to body size. Reproduction may be the most sensitive measurement in resident fish, and is of high ecological relevance. Changes in reproductive investment can be evident within a year (Bowron et al. 2009) since the reproductive tissue is generally turned over annually. Fecundity and gonad weight are easy to measure if an appropriate sampling time is chosen (Barrett and Munkittrick 2010). In terms of a change in gonad size, additional work related to magnitude could be focused on determining whether the change occurs at other times of the year (for multiple spawners). Studies related to magnitude could also focus on asking whether the changes are present in other species in the same exposure area.

Measures of stored energy reserves provide valuable information on the efficiency, availability, and quality of food available to the fish. The EEM program uses condition (length to weight relationships) and liver size as indicators for this information. As with other indicators, the consistency in response between indicators is important. Liver size can increase for several reasons, including storage of lipids and glycogen, and enhanced detoxification activity.

Analysis of data shows that there is a high amount of overlap in the information provided by condition and liver size (Fig. 1). Multidimensional scaling ordination of the benthic and fish responses from Cycle 2 of the pulp and paper EEM clearly show that fish population endpoints and benthic endpoints in the program give different information, as evidenced by the differences in the orientation of the principal component correlation vectors. It also shows that gonad size and age pull in opposite directions (i.e., smaller gonads pull in the same direction as younger-aged fish, and vice versa). Differences in the relative responses can be used to highlight response patterns (Hewitt et al. 2005).

**Basic Study Design Concerns**

**Reference Sites**

The most common study designs for fish population health assessments are control/impact designs and gradient designs. It is now common in these assessments to use multiple reference sites. Including additional reference sites increases the ability to evaluate issues related to natural variability, ecological relevance, and confounding factors, and improves the ability to evaluate the adequacy of the chosen reference site(s). However, the increased cost of using multiple reference sites has limited the implementation of better study designs. Over the first three cycles of monitoring in the pulp and paper EEM program, there has been a trend towards using more reference sites. In Cycle 1, 3% of studies used multiple reference sites, in Cycle 2, 9%, and in Cycle 3, 25% used multiple reference sites.

The choice of reference area is a typical criticism of studies. Ideally, the reference site in riverine assessments would be located upstream of the stressor, in similar habitat, free of confounding influences, with a natural barrier that limits movement between sites, but this situation is seldom available. There are several main issues involved in the selection of a reference site, including whether the site is comparable in terms of habitat, is free from the stressor of concern (exposure) and from confounding influences, or is open to movement of fish from the exposure site (fish in an upstream reference area could have been exposed previously or fish in the exposure area could be transient, reducing exposure to potential effects).

Studies that use a gradient approach and multiple reference sites are stronger than studies that depend on a single reference site. There are several new approaches to trying to deal with problems with reference sites, including reference condition approaches (Bailey et al. 1998) and using negative reference sites (using the exposed site as your reference; Vallières et al. 2007). Regardless, the existence of consistent changes over time increases the level of confidence that the changes are real. Follow-up studies must evaluate the adequacy of the reference site(s) especially if consistent results are not found.

**Fig. 1.** Multidimensional scaling ordination of Cycle 2 EEM pulp and paper fish and benthic invertebrate effect endpoints with principle component correlation vectors added. Fish endpoints: Age; G = gonad weight; K = condition; L = liver weight; WA = weight-at-age. Benthic endpoints: Ab = abundance; BC = Bray-Curtis Index; E = evenness; TR = taxon richness. Figure adapted from Lowell et al. (2003).
Confounding Factors

In the second cycle of Canada’s pulp and paper EEM program, almost 80% of the studies that concluded there were effects also recorded the presence of confounding factors in their interpretation of effects (Lowell et al. 2003). A long list of potential confounding factors exist at most sites, including other outfalls, habitat changes, historical uses and contamination, tributaries and nonpoint source inputs, among many other factors. In highly confounded situations, alternative methods should be considered, but it should be emphasized that it is possible to get interpretable field results at most sites with adjustments to the study design. There is no consensus as to what constitutes sufficient data to demonstrate that other discharges or contaminant sources are primarily responsible for observed changes or absence of observed changes. If changes are seen and thought to be potentially contributed to by confounding influences, the objective of subsequent study designs should be to eliminate the confounding influences as being a significant contributor to the issue.

Ecological Relevance of Endpoints Used

There are ongoing concerns expressed about the ecological relevance of the endpoints used in fish population health assessments. Changes in indicators of growth, reproductive performance, age distributions, and body condition are of ecological relevance, but the concern expressed by some is that the relevance of changes in these indicators to the population levels is unknown. While it is possible to mathematically model the population consequences (e.g., Miller et al. 2007; Watanabe et al. 2007), such modelling efforts ignore site-specific factors that determine the population-level consequences of change, including mobility, refugia, species interactions, and seasonal changes in environmental conditions.

Decisions about measurement endpoints require consideration of factors related to the time scale of responses, their reversibility, sensitivity, and their ability to be linked to causative agents. There is general agreement that the loss of an important fish species or the presence of contamination that affects the consumption potential of fisheries resources are changes that are important to people. The retrospective assessment process described here is meant to provide some information on how close we might be to thresholds where we need to become concerned before an important species is lost.

Decisions regarding monitoring level (community, population, individual, biochemical) are a compromise between conflicting concerns over ecological relevance and ease of detection with those of time scale of response, reversibility, and ability to identify cause. While many jurisdictions and studies focus at the community level (e.g., Hall et al. 2009; Karels and Niemi 2009), the Canadian EEM program did not focus on this level because of a variety of issues, including the difficulty in nationally standardizing an approach, the iterative nature of the program, effort and cost of good community programs, and analytical concerns over the seasonality and ability to isolate the causes of any changes detected.

In Canada, the Fisheries Act requires that we protect fish, fish habitat, and human use of the fisheries resource. The EEM program addresses those three aspects using the adult fish survey (discussed here), an invertebrate community survey (to assess fish habitat), and fish tissue studies (human use of fisheries). Within the program, additional methods are incorporated following the confirmation of effects of the discharge in the investigation of cause and solutions portion of the program or for more research-related studies.

Other jurisdictions (such as Sweden) incorporate impacts at biochemical, individual, population, and community levels (Swedish EPA 1997). This was not done in Canada because of the difficulty in incorporating effect sizes and thresholds at the biochemical level for all of the more than 60 species (Barrett and Munkittrick 2010) that have been used across the country, and at the community level because of the reasons outlined above.

Natural Variability

Natural variability is the tendency for endpoint values to change spatially and/or temporally from nonanthropogenic causes. Natural variability is contributed to by a number of components, including annual variability in food availability, habitat quality (flow, temperature, etc.), as well as annual variability attributable to sampling design (sampling gear or bias, changes in personnel and training, measurement error, or equipment performance).

There is some overlap and confusion regarding the concerns about adequacy of the reference site, ecological relevance, and natural variability. Natural variability refers to the chance that a specific site difference relative to a specific reference site would reverse in a different year. Concerns about the adequacy of the reference site are better described by concerns about whether an effect reflects a true difference associated with exposure to the stressor(s) of interest, and the chance that a different interpretation would occur with the use of a different reference site. The issue of ecological relevance (within this context of variability) refers to the size of the effect relative to the values that could be seen between a variety of reference sites. A difference smaller than what is seen between numerous reference sites is not interpreted to mean that the site-specific difference is not real relative to a local comparable reference site, but it does provide input into the ecological importance and the relevance of the difference.

Statistical Design Issues

Statistical design issues can be divided into three main criticisms reflecting concerns over the number of comparisons made, power analysis, and pseudoreplication.
There have been many expressed concerns about multiple comparisons. In the EEM program a significance level of $\alpha = 0.05$ is used at the mill or mine level for each comparison. There were several safeguards put into place: progression to a new tier of monitoring only occurs if an endpoint responds above a CES, that effect is confirmed in a subsequent cycle of monitoring (three years later) in the same direction, and that related endpoints are responding together in an interpretable pattern (eutrophication, food limitation, metabolic disruption). Furthermore, analyses of responses have shown that responses are usually confirmed in the opposite sex, or a second species (two fish species are required for monitoring in the EEM program), and that responses seldom occur individually (<8% of the time).

There has been some debate over the levels of $\alpha$ (the probability of committing a type I error) and $\beta$ (the probability of committing a type II error) in EEM studies. In earlier cycles of EEM, $\alpha$ was set at 0.05 and $\beta$ was set to 0.20 (equivalent to a power level of $1 - \beta = 0.80$). The EEM program now recommends that $\alpha$ and $\beta$ equal one another, letting the risk to the environment (probability of committing a type II error) equal the risk to industry (probability of committing a type I error). If values are set at $\alpha = \beta = 0.10$, the sample sizes required to detect the same effect are approximately the same as when $\alpha = 0.05$ and $\beta = 0.20$. Where possible it is encouraged to reduce $\alpha = \beta = 0.05$ (the traditional level for $\alpha$). These recommendations are to help ensure that studies are designed to provide a reasonably high probability of statistically detecting a predetermined effect size (ES) if it has occurred (i.e., the power of the test $(1 - \beta)$ should be high).

Briefly, pseudoreplication (Hurlbert 1984) arises because it is not possible to randomly assign field sites, and the exposure treatment (exposure site) cannot be replicated. In a river system, it has been shown that, where possible, upstream and downstream data are essential for interpreting the effects of the effluent (Munkittrick et al. 2000). But in these situations, the reference site must be upstream and the exposed site must be downstream (except in unique cases). Since the sites cannot be randomly assigned, there may be factors that have not been controlled (i.e., factors other than timing, sample gear, habitat type etc.) that may account for the differences between sites. Also if a significant difference is detected, this provides evidence that there is a difference between two locations (a site downstream of the effluent and a site upstream). This difference cannot be interpreted as a difference resulting from the stressor of interest, but only as a difference between two specific locations.

EEM studies focus on trying to develop an understanding of that specific reach, so that site-specific concerns can be identified, and necessary changes can be made on a local basis. Monitoring is conducted over time and consistent significant responses over time are used to direct more focused monitoring to uncover the course of the impact. The ability to extrapolate the results in most studies is not important. Unless there is another site with identical stressors and similar habitats, extrapolation will seldom be possible. It is not possible with data from a single site to make wide conclusions. It must be restricted to comment on one situation and design hypotheses to test at others. The best approach is based on developing an iterative understanding (Hodson et al. 1996). The conclusion of a round of monitoring should only be used to design subsequent monitoring steps which ultimately lead to uncovering cause, thus the design must be iterative. The repeated observation of consistent changes over a longer time period will increase confidence that the changes are real and interpretable as differences between sites. An observed change of decreased food availability at a site can equally be interpreted as increased food availability at the reference site. Iterative studies must be designed and used to focus studies to test the validity of the conclusion.

Wide conclusions about the impacts require analysis of data from multiple sites (e.g., Lowell et al. 2005). With pulp and paper impacts, the studies were initiated in Canada at a single mill (Munkittrick et al. 1991), expanded to eight mills (Munkittrick et al. 1994), and then went to data from 65 mills (Munkittrick et al. 2002; Lowell et al. 2005). Meaningful information and industry- and country-wide conclusions can be generated with data from 65 mills, but many monitoring programs will need to make decisions based on a minimal number of reference sites.

**Potential Genetic Influences**

Potential genetics issues include whether the absence of a difference between sites represents genetic adaptation, whether differences between populations are genetically-based and natural, and whether reductions in genetic variability are an important response on their own. If there is no detectable difference between sites, then for the purposes of evaluating sustainability, the conclusion is that the situation is sustainable, regardless of the mechanism that led to it. If differences exist that are genetically based, the iterative sampling program will eventually lead to a conclusion that the differences between sites are independent of the stressor, and that the situation for the population is sustainable.

The issue of reducing the genetic variability of exposed populations is real (e.g., Bell and Collins 2008) and is thought to represent increased vulnerability of the population. The issue is whether the situation may be close to an unidentified threshold that is only evident through biochemical or chemical measurements, and this issue of potentially more significant lower thresholds is currently beyond the scope of EEM.

**Selection of Species and Timing of Sampling**

The most important factors when selecting fish species for assessing the health of fish populations are exposure,
abundance, relevance to the study area (Munkittrick et al. 2000), and sensitivity to the effluent. The recommended method for carrying out a fish population survey is to monitor adults (sexually mature) of relatively sedentary finfish that have been exposed to effluent over a long period of time. In selecting a species, the species selected for previous population health studies should be considered if possible, and preference should be given to: resident (nonmigratory) fish species identified in site characterization, sexually mature female and male fish species that are abundant in both the exposure and reference areas, fish species for which fishing or sampling permits can be obtained, and fish species that have the highest exposure to effluent.

Some of the challenges related to species selection relate to the attempt to design a single program for multiple purposes. It is very difficult to accomplish this. Concerns about contamination of fishery resources for human consumption would direct the study design to collect a species that is long-lived (so that contaminants can accumulate over a longer timeframe), is piscivorous (so that biomagnification is higher), matures late (to increase concentration), preferably focuses on male fish or species that do not spawn every year (so that elimination of contaminants through egg deposition is lessened), and are of importance for local consumption. These characteristics are exact features that decrease the sensitivity for detecting environmental impacts, where the preference is for species that are benthic (because generally they are less mobile), are not commercially or recreationally important (because it obscures determining cause), mature early, contribute a lot of energy to reproduction (so that energy demands are high), and are short-lived (so impacts are recent), and the focus is on female fish (environmental impacts are often more serious on female egg producers since female gametes are typically limited relative to male gametes).

There are a number of other factors that need to be considered when selecting a sentinel species (see Munkittrick and McMaster 2000; Munkittrick et al. 2000), including ensuring that the species are active participants in the local aquatic food web. Other life history characteristics, like spawning time and migration, need to be evaluated site-specifically because the interaction between discharge site, spawning habitats, seasonal changes in flow, and dilution all play a role in influencing the importance of the characteristics and how they potentially impact the sensitivity of the monitoring program.

The trend towards the increasing use of forage fish species (Munkittrick et al. 2002) has continued in the EEM pulp and paper program, rising from their use in 10% of surveys in Cycle 1, to 26% in Cycle 2, and 34% in Cycle 3. Their use has several advantages and disadvantages. On a practical level, small-bodied fish species are usually more abundant, easy to capture, and more sedentary than larger-bodied fish species. Shuter (1990) noted that small-bodied fish species were more sensitive to acidification than larger species, or at least responded more quickly to changes in pH. In freshwater fish, home range size has been positively correlated with body size (Minns 1995), and many small-bodied species integrate local conditions very well. On the other hand, they require more sensitive analytical balances, more careful measurement, and are more sensitive to microhabitat differences because they integrate the local habitat so well. They are also more sensitive to differences in timing of sampling (Barrett and Munkittrick 2010).

The suitability of fish as sentinels depends on a number of factors, but the timing of sampling is very important. A variety of factors needs to be considered, including potential migratory behaviour of the sentinel species, water conditions (flow, turbidity, wave action), accessibility, and the cycle of gonadal development for the sentinel species. If historical data exists, it would be useful to examine that data and, if appropriate, conduct the survey during similar periods so that the surveys can be compared. Similarly, subsequent monitoring should be conducted during similar periods of the year to be comparable if that sampling time makes sense for the study. Barrett and Munkittrick (2010) have developed species-specific sampling times for the more than sixty different species that have been used in EEM studies in Canada. The sampling times are based on the reproductive strategies and cycle of gonadal development for each species and are provided in Table 2 along with some species-specific aspects that may affect study designs.

**Nonlethal Sampling Methods**

Nonlethal sampling of fish can also be used to evaluate the effects of stressors on fish populations. Sample size requirements for these studies are recommended to be a minimum of 100 fish older than young-of-the-year (YOY), and an additional 100 YOY fish from each study site. It is usually possible to separate YOY from older age classes by size distributions; however, this may not be possible for species with extended spawning periods. If YOY abundance is extremely high (>80 to 90%), the proportion of fish that are YOY should be estimated from the first 100 fish collected, and then the collection can continue concentrating only on collecting 100 larger individuals for calculating size distributions of older fish. The fish collected that are older than YOY should represent the whole range of fish sizes and be representative of the population (mature and immature). When YOY are abundant and constitute a high relative proportion of the population, there will not be sufficient information collected on all other size/age classes. In the latter two situations, the collection of the additional non-YOY fish allows for a higher discrimination of the older fish classes to be achieved. The use of this recommended sample size in each area will give a good idea of the population distribution when plotting endpoints such as the length-weight frequency. As well, when examining differences between the relative abundance of young
<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Scientific name</th>
<th>Considerations</th>
<th>Sample time</th>
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</table>

*TABLE 2 continued on next page*
versus mature fish, fairly good resolution is achieved (Gray et al. 2002).

Fish for nonlethal sampling should be measured for length and weight, and external sex determination should be made if possible (Gray and Munkittrick 2005). If only adults are used, the priority should be to sample prior to or at the start of the spawning season. However, if YOY are to be collected, the timing should move to the late fall when it will be easier to measure YOY for most species (spring spawners).

The size distribution should be examined as a surrogate for differences in age. If a site difference is present, subsequent monitoring should focus on understanding the difference and possible causes. Size distributions can be analyzed by the Kolmogorov-Smirnov test, although this test is very conservative. There are challenges to using age information on many short-lived species of fish. If a fish only lives two or three years, it will not be possible to measure a 25% difference in mean age (CES).

### TABLE 2 continued

<table>
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<td></td>
<td>Torrent sculpin</td>
<td><em>Cottus rotheus</em></td>
<td>S/G LM</td>
</tr>
<tr>
<td></td>
<td>Spoonhead sculpin</td>
<td><em>Cottus ricei</em></td>
<td>S/G LM</td>
</tr>
<tr>
<td></td>
<td>Shorthorn sculpin</td>
<td>Myoxocephalus scorpius</td>
<td>W/G</td>
</tr>
</tbody>
</table>

*a* First letter or characters separated by a slash(es) (/) represent “SPAWNING;,” and the subsequent characters represent “MIGRATION” and “OTHER” considerations as follows:

**SPAWNING**

S = spring or early summer spawners, usually a single spawning event per year, usually migrate to spawn.

F = fall spawners, usually single spawners.

W = winter spawners, usually single spawners.

X = Multiple spawners – usually summer spawners, and need to worry about whether small fish are reproducing less (or more) than larger fish (view GSI versus length), variability will increase as approach spawning season.

G = males are guarders – reproductive investment in males will be low, but are likely not very mobile.

A = asynchronous spawners.

? = some doubt about number of spawns.

**MIGRATION**

M = Mobile – are known to migrate considerable distances (or change habitats significantly) for spawning at some sites.

* = may be site-specific.

M? = probably migrate.

A = anadromous forms may be present, and may be much larger and much more mobile.

C = Catadromous, migrate out as adults.

R = research available to show that they are usually resident.

**OTHER**

P = planktonic feeder – migratory feeder.

1-yr = 1-year life cycle.

2-yr = 2-year life cycle.

H = juvenile and mature fish may utilize very different habitats.

LM = females may start to mature very late in the year, fish may spawn during fresheret – can be difficult to get mature female gonads.

D = diet changes within the size of sexual maturity to piscivorous at larger sizes (when available)

*b* Sampling times adapted from Barrett and Munkittrick (2010); 4–6 = 4 to 6 weeks prior to spawning; LF = late fall (before ice cover); SPAW = as close to the first spawning event of the season as possible.
It should be possible at most sites to get an estimate of growth and reproductive success using nonlethal methods. Growth can be evaluated by the size of YOY at the end of the growing season, and by the size of fish older than YOY. A comparison of the size of YOY fish gives a good indicator of growth since it is a direct indicator, in comparison with size-at-age which is indirect. Differences between sites in spawning times will be integrated into this endpoint. It is also possible to get a growth estimate by a shift in size distributions over time (i.e., repeating measurements two months apart at the same sites), or differences in average size. If the fish species chosen is externally sexually dimorphic, it is possible to examine whether there are gender-specific differences in growth rate.

Reproductive success can be assessed using relative age class strength or by the relative proportion of YOY individuals (Gray et al. 2002). A length frequency distribution may be plotted as a surrogate of an age frequency distribution. Size frequency analysis can be used to examine age distributions, size-at-age data, and condition factors for the fish (Gray et al. 2002). It is recommended that, if possible, aging structures be sampled from a subsample of each size class for situations where age may need to be verified. In slimy sculpin (Cottus cognatus), it has been found that there is rapid growth of YOY fish in the spring, which can cause some overlap with fish older than YOY, making resolution difficult (Gray et al. 2002). Thus, length frequency distributions may be easier to make on late summer and early fall data.

Condition factor can also be evaluated by the relationship $K = 100,000 \times \frac{\text{weight}}{(\text{length})^3}$ of the fish examined (when weight is in grams and length is in millimetres). A large number of areas can typically be sampled using this approach, and it is encouraged to sample multiple exposure and reference areas.

**Sample Size Requirements, Effect Sizes, and Power**

Munkittrick (1992) suggested that 15 to 25 female fish of a properly selected species and size range will yield sufficient information to characterize a population. Data from more than 300 fish population surveys from the EEM pulp and paper program have been examined to determine the statistical power to detect a 25% CES in the EEM effect endpoints (10% difference for the condition endpoint) when sample sizes were between 15 to 25. The distributions of observed power levels by endpoint are provided in Fig. 2. In general, analyses of condition, relative liver weight, and weight-at-age had high power levels to detect an effect equal to the respective CES. The relative gonad weight and age comparisons are typically more variable than the other endpoints and thus these comparisons have lower power to detect effects. The proportions of comparisons with power levels greater than 0.8 by endpoint are 78, 58, 49, 61, and 42% for condition, relative liver weight, relative gonad weight, weight-at-age, and age, respectively. Based on these power levels, a sample size of 15 to 25 per sex and species seems reasonable for a preliminary evaluation, and variability from preliminary studies can be used in power analyses to determine sample sizes required in follow-up studies.

An extensive literature review has shown that CESs which have been defined in other programs are often consistent with a CES of around 25% or two standard deviations for many biological or ecological monitoring endpoints, and this value appears to be reasonable for use in a wide variety of monitoring programs and with a wide variety of endpoints (Munkittrick et al. 2009). Barnthouse et al. (1989) argue that a 10% change in variables would be societally and ecologically significant, although they were concerned primarily with laboratory toxicity tests and not field surveys. Their proposed CES was deliberately conservative (small) because of concerns about the uncertainty in extrapolating laboratory results to the field (Environment Canada 1998). The EEM program currently uses CESs of 25% for all fish population endpoints except for condition which has been set at 10%. These values were chosen primarily on data distributions of observed effects from within the program.

When preliminary analyses show that power will be insufficient given reasonable sample sizes, the assessments should be redesigned. Studies are designed site-specifically and the priority should be given to reducing variability rather than increasing sample size. CESs in the EEM program are defined as percentages of the reference mean and are not represented in the measurement units of the response variable, as these CESs would vary for different studies; therefore the coefficient of variation (COV), expressed as a percentage of the reference mean, is used as a measure of variability in sample size calculations (COV = standard deviation / reference mean × 100%). For a basic control/impact ANOVA (analysis of variance) design with untransformed data (e.g., as used for the age endpoint in EEM studies), the estimated sample size required to detect a given ES (effect size) at a given power level can be calculated as follows (Green 1989):
\[ n = 2(t_{\alpha} + t_{\beta})^2 \left( \text{COV}/\text{ES} \right)^2 \]

(1)

where \( n \) is the sample size required at each site, \( t_{\alpha} \) and \( t_{\beta} \) are the values of Student’s \( t \) statistic (two-tailed for \( t_{\alpha} \) and one-tailed for \( t_{\beta} \)) with \( n - 1 \) degrees of freedom at a significance level of \( \alpha \) and \( \beta \), respectively. \text{COV} and \text{ES} are each expressed as a percentage. The sample size equation is solved iteratively by choosing an approximate value of \( n \) to start with (usually 20).

For a basic control/impact ANCOVA (analysis of covariance) design using log transformed data (e.g., as used for the relative gonad weight endpoint), the estimated sample size required to detect a given ES at a given power level can also be calculated by using a different version of equation 1. This equation is as follows (Green 1989):

\[ n = 2(t_{\alpha} + t_{\beta})^2 \left( S_z/\Delta_x \right)^2 \]

(2)

where \( S_z \) is the standard deviation of the residuals using log transformed data and \( \Delta_x = \log(f + 1) \), where \( f = \text{ES} \) represented as a fraction of the reference mean (e.g., ES of 25% \( \Rightarrow f = 0.25 \)).

Unusual Observations, Statistical Assumptions, and Data Analysis

Barrett et al. (2010) discuss unusual observations in the EEM fish survey data. These observations can occur for a number of different reasons and should only be removed under certain circumstances. Outliers can occur when a test subject that does not belong to the population of interest is included in the study; outliers should be removed if they can be correctly identified. Obvious data entry errors should be corrected if possible and naturally occurring large or small observations should not be removed. Comments associated with unusual observations should be recorded in the field upon sampling to help determine whether data should be removed from an analysis. It is recommended to perform two separate analyses: one containing all data, and another with any outliers removed to determine the consequences of removing the data and to allow the reader to decide whether the observations should be removed or not.

The inclusion of immature fish data in statistical analyses can provide misleading results. Immature fish devote proportionally more energy towards growth, and have varying body size and gonadal growth relationships. For data analysis, fish identified as immature should be removed. The gonadosomatic index (GSI = gonad weight / body weight \( \times 100\% \)) can be useful in identifying immature fish. For many fish species, immature fish can be identified as having a GSI of <1%. A plot of gonad weight versus body weight can also be useful in identifying immature fish. Comments from field observations may also assist in identifying unusual data that are suspected to be from immature fish. If immature fish are being used in any data analyses, it should be in comparing immature fish to immature fish between sites.

The standard statistical assumptions required for many parametric statistical tests are those of independence, normality, and homogeneity of variances. The two parametric analyses used to analyze the EEM fish population endpoints are ANOVA and ANCOVA. The assumptions for these tests are discussed below.

Independence (Pseudoreplication).

When designing experiments, it is desirable to ensure that replicates are randomly allocated to different treatment levels such that the response of each replicate is independent of other replicates. This element of randomness provides some assurance that observed differences in responses among treatments result from treatment effects and not for some other reason. Randomly allocating replicates to different treatment levels is a relatively easy procedure when conducting manipulative experiments (e.g., controlled laboratory tests), but is less obvious for observational field studies. Observational studies, such as environmental impact studies (e.g., single stressor EEM studies) or environmental assessments (i.e., multiple stressors), test hypotheses about the presence and magnitude of effects. However, the strength of inferences from these types of experiments is limited for two reasons (Paine 1990): the stressor (e.g., mill outfall, hydroelectric dam) cannot be replicated, and stressors cannot be applied randomly to replicates. What this means is that the stressor or treatment is always partly or wholly confounded with space or time and that the observed effects may or may not be caused by the stressor of interest. When significant differences are observed between reference and exposed fish populations, it is only possible to conclude there are differences between these two populations, not that the differences were caused by effluent exposure. Interpreting significant differences as treatment effects when either treatment is not replicated or replicates are not independent is referred to as pseudoreplication (Hurlbert 1984), as discussed previously.

It is critical that observations be confirmed, through replication over time, and that some effort be expended to confirm that the stressors of interest are involved in the responses before attributing cause to any specific stressor.

Normality and Homogeneity of Variances

The assumptions of normality and homogeneity of variance should be assessed before applying parametric procedures. However, most univariate normal distribution-based statistical methods are quite robust and can support moderate violations of the assumptions. Transformation of original data will help normalize the data or homogenize the variances. Logarithmic transformations are often preferred because most biological measures are considered to operate on a log or exponential scale (Peters 1983), and such a transformation
is biologically meaningful. If the transformations are unable to produce data that meet the assumptions, then a plot of the residuals may reveal problematic data points that may warrant investigation. Most of the univariate statistical methods are robust under moderate violations of assumptions, with some exceptions, including analyses with small and unequal samples. For serious violations, nonparametric statistics can be considered.

Additional Assumptions

An assumption of ANCOVA is that the independent variable (covariate) is fixed and measured without error. This assumption is frequently violated and Draper and Smith (1981) discuss the consequences of this violation. This is likely to prove problematic only in situations where the range of the independent variable is very small. The assumption of a linear relationship can be tested for samples with multiple observations at different values of the independent variable. This may be possible for discrete variables such as age, but not for continuous independent variables such as body weight (Environment Canada 1998).

An additional assumption of ANCOVA is that the regression slopes of each treatment (site) are parallel. When this assumption is not met, the ANCOVA procedure cannot proceed since there is a covariate by treatment interaction, and differences in the response variable among treatments vary at different values of the covariate. There are a few options for dealing with nonparallel regression slopes in ANCOVA that are useful in analyzing fish population survey data. Barrett et al. (2010) provide two different methods for dealing with data sets with nonparallel regression slopes so that they can be analyzed using the parallel slope ANCOVA model. The first method identifies data sets where the slopes are forced nonparallel by high-influence observations, which can be removed to provide parallel slopes. The second method identifies data sets where a model with nonparallel slopes is statistically, but not practically, significant and ANCOVA can proceed using the parallel slope regression model. Lowell and Kilgour (2008) provide another method for analyzing data when regression slopes are not parallel. They estimate the effects for smaller (or younger) and bigger (or older) fish by calculating the difference in the response variable at the values of covariate where the ranges for each site overlap. These estimates can then be compared to CESs.

It has been suggested that the range of the covariate in ANCOVA should be approximately the same for each site. This will be difficult to assure in practice, but the violation of this should be considered when interpreting results from such cases. If there is reason to believe that there are issues with the overlap of the range of covariate values, then a single factor ANOVA can be performed on the covariate values between sites. If the covariate means do not significantly differ between sites, then the results of the ANCOVA will probably be reliable (Quinn and Keough 2002). A significant difference in the mean covariate values between sites is on its own a significant effect. Things to consider would be the consistency of sampling gear between sampling sites and selection of samples in interpreting differences in the covariate means or ranges observed. It may be appropriate to provide an analysis on a subset of the data, omitting unusually high or low covariate values to provide a reliable analysis. Also, for several small-bodied fish species the range of the covariate (age) might only be 2 and 3 or 2, 3, and 4. An ANCOVA with only two or three values of the covariate can provide misleading results. In these cases it is appropriate to perform a one-factor ANOVA on body weight using site as the factor for each age group.

ANOVA and ANCOVA are robust to violations of the assumptions of the test when sample sizes are equal (Huijema 1980; Hamilton 1977) but quite often unequal sample sizes are prone. When assumptions are seriously violated and sample sizes are unequal, nonparametric alternatives to these tests could be considered. The Kruskal-Wallis test on the equality of medians is an appropriate alternative to ANOVA, and several different nonparametric techniques have been proposed for ANCOVA using ranks. Conover and Iman (1982) proposed a nonparametric alternative in which the response and the covariate are replaced by their ranks. The analysis is the same as the parametric ANCOVA using the ranks as data and is the simplest nonparametric alternative. Some other nonparametric alternatives to ANCOVA are discussed in Shirley (1981) and Quade (1967).

Conclusions

Monitoring fish at the population level gives a balance between the ecological relevance of changes at the community level and the sensitivity of responses at the biochemical level. Changes at the population level are important to monitor for the protection of fish species. Since the implementation of the pulp and paper EEM program in 1992, there have been many advancements along with new research available to improve fish population health assessments. The guidance provided in this paper can be used to improve study designs and the interpretation of fish surveys.

It is important that monitoring of fish populations is conducted over time so that observations can be confirmed and follow-up studies can be designed to address issues of concern. Life history characteristics of sentinel species should be studied and species should be selected to best answer the questions of the monitoring program. Monitoring should be conducted at an appropriate time to assess the questions of the monitoring program. Usually 15 to 25 fish of each species and sex are sufficient for preliminary assessments, and power analyses can be used to determine sample sizes for subsequent monitoring. Nonlethal sampling methods have been developed and should be considered if the methods can be used to answer the questions of concern. Careful data quality assurance / quality control and data analysis are necessary to obtain reliable results and design subsequent monitoring.
References


Barrett TJ, Munkittrick KR. 2010. Seasonal reproductive patterns and recommended sampling times for sentinel fish species used in environmental effects monitoring programs in Canada. Environ. Rev. 18:115–135.


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Physiological Health of Common Bully (Gobiomorphus cotidianus) in the Tarawera and Rangitaiki Rivers of New Zealand: Evidence of Diminished Ecological Effects of Pulp and Paper Effluents in Wild Fish Populations

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This study examined the comparative physiological health of the endemic New Zealand common bully (Gobiomorphus cotidianus) in the Tarawera and Rangitaiki Rivers. Bullies were sampled downstream of pulp and paper effluent inputs in the Tarawera River and compared with a similar inland population in the Rangitaiki River. Condition factor and liver somatic index did not differ between populations, but Tarawera River bullies possessed larger gonads. Haematological assessments found smaller erythrocytes with reduced haemoglobin content, and increased leukocyte concentrations in Tarawera River females. Male and female Tarawera River bullies had significantly induced hepatic ethoxyresorufin-O-deethylase (EROD) activity. Greater ovarian follicular steroid production was also found for Tarawera River females. Microscopic analysis of ovarian tissue samples found no histopathological abnormalities in either population and indicated a slightly advanced vitellogenic stage of development in the Tarawera River population. It is concluded that the difference in steroid production between populations was most likely linked to gonad size and developmental status. In line with the disappearance of physiological effects in recent controlled laboratory and mesocosm pulp and paper effluent exposures, this study further demonstrates that, with the exception of EROD induction, characteristic pulp and paper effluent effects are not obvious in wild Tarawera fish.

Key words: pulp and paper effects, reproduction, fish, Gobiomorphus cotidianus, New Zealand

Introduction

International research on the environmental effects of pulp and paper mill effluents has been ongoing for over 40 years. Considerable effort has been directed at determining and understanding the physiological effects of effluent exposure in fishes. The ability of various mill effluents to alter reproductive physiology in particular has been well established (Munkittrick et al. 1997; Hewitt et al. 2008). Although effects continue to be observed in both laboratory and field settings (McMaster et al. 2006; Parrott et al. 2006), at least some degree of recovery in wild populations has been observed in Canadian mill effluent receiving environments over the last decade (McMaster et al. 2006).

The majority of pulp and paper effluent effects-based research in New Zealand has focused on the Tasman and Kinleith pulp and paper mills that respectively discharge their effluents into the Tarawera and Waikato Rivers of the North Island. The most intensive research program has focused on the Tasman Mill and Tarawera River, which through controlled laboratory and mesocosm studies, has revealed the gradual disappearance of physiological effects that have been linked to mill process changes and improved effluent quality (reviewed by van den Heuvel et al. 2010). In contrast to controlled Tasman Mill effluent studies, the predominately wild fish monitoring program on the Waikato River has found that population and reproductive impacts continue to be observed within the pulp and paper mill effluent-impacted zone of this system (West et al. 2006; Landman et al. 2008). Consequently, the question of whether or not similar physiological effects associated with prolonged effluent exposure may be occurring in wild Tarawera River fish populations remains largely unanswered.

The main factor preventing a physiological impact assessment of wild fish in the Tarawera River has been the inability to identify a suitable monitoring species (van den Heuvel et al. 2010). It was ultimately determined that the endemic New Zealand common bully (Gobiomorphus cotidianus) would be the most suitable candidate since this species is known to exhibit high site fidelity as adults, thus reflecting local environmental conditions, and their benthic nature ensures exposure to both water and sediments within a given system (West, 2006; van den Heuvel et al., 2007; Landman et al. 2008; Bleackley et al. 2009). However, the first attempt to undertake a physiological assessment of this species in the Tarawera River revealed genetically distinct and reproductively asynchronous upstream and downstream populations.
preventing comparisons from being made between effluent-exposed and unexposed fish (van den Heuvel et al. 2007). It was not until a genetically, reproductively, and geographically analogous population was confirmed in the Rangitaiki River (Michel et al. 2008; Bleackley et al. 2009) that a physiological impact assessment of wild fish in the Tarawera River could be undertaken.

This study sought to examine the physiological effects of long-term pulp and paper effluent exposure of resident common bully in the Tarawera River downstream of mill effluent inputs through a direct comparison with an unexposed reference population of similar geographic location (inland distance) in the Rangitaiki River. To attain a general view of physiological health, a selection of biological endpoints were employed which were directed across several levels of biological organization to include physiological (condition factor and organosomatic indices), reproductive (ovarian steroid biosynthetic capacity), histopathological (blood, ovary tissue), and biochemical (hepatic mixed function oxygenase activity) endpoints.

Materials and Methods

Study Sites and Mill Descriptions

This study examined fish populations in the Tarawera and Rangitaiki Rivers in the Bay of Plenty Region of the North Island, New Zealand (Fig. 1). The Tarawera River has a watershed area of 984 km² and originates from Lake Tarawera. The river travels a distance of approximately 55 km to the coast at Matata where it enters the Pacific Ocean. Land use in the upstream reaches of the river consists of predominately native and exotic forests, with very little human habitation (Rutherford 1997). In contrast, the downstream Tarawera River has been heavily modified following wetland drainage and river diversion and channelization. The Rangitaiki River runs parallel to the Tarawera River. It is a larger river with a total length of 155 km and a total catchment area of roughly 3,000 km². The headwaters of the Rangitaiki River originate in the central volcanic plateau within the Kaingaroa ecological district, and the river exits the coast at Thornton. The river has been hydrologically modified by the construction of two hydroelectricity dams located along its mid section. Land use within the Rangitaiki catchment is dominated by agriculture, particularly dairy farming, contributing to diffuse nonpoint source nutrient inputs along the river.

There are two separate pulp and paper mills operating alongside the Tarawera River at the township of Kawerau. The largest mill is an integrated bleached kraft (BK) pulp mill and thermomechanical pulp (TMP) and paper mill, hereafter referred to as the Tasman Mill. The kraft mill produces approximately 300 and 500 T/d of unbleached and bleached pulp, respectively. The paper mill produces 900 T/d of thermomechanical pulp and 1,000 T/d of newsprint paper. Mill furnish is approximately two thirds Pinus radiata softwood and one third Eucalyptus sp. hardwood. The Tasman Mill has been elemental chlorine free since April 1998, and kraft pulp now undergoes chlorine dioxide bleaching. The combined TMP/BK effluents are primary treated by passage through a gravity clarifier which removes course solids. Secondary treatment is through an aerated lagoon system with a hydrological retention time of 4 to 5 d, after which the effluent is discharged directly into the Tarawera River at a rate of approximately 130,000 m³/d. The dilution of effluent in the Tarawera River ranges from between 5 to 12% vol/vol, representing the single largest contaminant source by volume in this system.

The second mill is a tissue paper mill. This mill historically produced approximately 110 T/d of unbleached and peroxide bleached chemithermomechanical pulp and 160 T/d of tissue paper. Pulp mill wastewater in combination with Kawerau municipal sewage effluent was treated via an anaerobic system, discharging between 6,000 and 8,000 m³/d of effluent, both directly to the river and to river-side rapid infiltration basins. Production of pulp by the tissue mill was terminated in May 2007 just prior to this impact assessment. White water from the tissue paper-making process is first passed through a clarifier and then integrated into the Tasman Mill secondary treatment system before final discharge to the river.

Fig. 1. Location of the Tarawera and Rangitaiki Rivers in the Bay of Plenty Region, New Zealand. Key features including the combined pulp and paper effluent outfall on the Tarawera River and the paired downstream fish sampling sites are indicated by arrows.
Fish Sampling

The physiological health assessment of common bully in the Tarawera and Rangitaiki Rivers was performed toward the end of July 2007. Fish collection was timed just prior to peak gonadal growth in these populations as identified by Bleackley et al. (2009) so that reproductive endpoints could be measured. The Tarawera River site (DT) was located downstream of the Tasman Mill outfall and paired against a site of roughly similar inland distance in the Rangitaiki River (DR; Fig. 1). Approximately 40 adult fish (20 male, 20 female) per site were captured using Gee minnow traps set overnight. Captured fish were transported back to the laboratory in 20-L plastic pails within two hours of capture and held in aerated river water prior to necropsy.

Sampling Protocol

Fish were anaesthetised (0.1 g/L MS-222, Sigma-Aldridge) prior to measurement and sampling. Approximately 30 to 100 μL of blood was taken by caudal venipuncture using preheparinised (5,000 IU/mL) 0.5-mL tuberculin syringes and stored on ice until processing. Fish were individually measured for body weight (±0.01 g), total length (±1.0 mm), organ mass (liver, gonad, spleen; ±0.001g), and carcass weight (±0.01 g). Livers were removed, weighed, and frozen in liquid nitrogen for biochemical analysis. Single ovaries were placed in histology cassettes and preserved in 10% neutral buffered formalin. Subsamples of whole ovaries from selected fish (size permitting) were placed in histology cassettes and paired against a site of roughly similar inland distance (DT) was located downstream of the Tasman Mill outfall identified by Bleackley et al. (2009) so that reproductive endpoints could be measured. The Tarawera River site was measured using the longest dimension of the follicle.

Haematology

Blood samples were analyzed for haematocrit, haemoglobin, total erythrocyte count, mean cell haemoglobin, mean cell haemoglobin concentration, mean cell volume, and total and differential leukocyte counts within 30 minutes of sampling. Standard methods were employed for the determination of most haematological endpoints (Dacie and Lewis 1991). Total erythrocyte and leukocyte counts were determined using recently developed flow cytometric methodology (Taylor 2009). Briefly, anticoagulated whole blood (10 μL) was suspended in a TruCount tube (BD Biosciences) containing 3,986 mL of Minimum Essential Medium (Gibco) with 0.25% bovine serum albumin (Sigma). The TruCount tube contained an accurately known predosed number of fluorescent beads. Immediately after cell dispersion, 4 μL of 0.5 mg/mL dihexyloxacarbocyanine, DiOC6(3) (Molecular Probes) in dimethylsulphoxide (Sigma) was added, and then the sample was incubated on ice for 30 min in the dark. Flow cytometry counts were performed on a FACSVantage SE DiVa flow cytometer (BD Biosciences) equipped with a 488-nm laser powered at 300 mW. Forward scatter (FSC), side scatter (SSC) and fluorescence were measured in the 530/30 nm wavelength range (FL1). The detector photomultiplier voltages were set at 200, 300, and 500 mV, respectively, and were viewed in logarithmic mode. Threshold was set at channel 1,000 on the FSC detector. Sample flow was adjusted to yield a count rate of 2,000 events/s. Data was displayed on SSC versus FL1 dot plots, and gates were set around the erythrocyte, leukocyte, and fluorescent bead populations to define each group. A total of 500 fluorescent beads were counted. Cell count per millilitre for erythrocytes and leukocytes was determined using the following formula:

\[
\text{cells/mL} = \frac{\text{cell count} \times \text{total bead count (TruCount tube)}}{\text{actual bead count (500) \times \text{blood volume (0.01 mL)}}
\]

Differential leukocyte counts were determined through the examination of blood smears prepared using 2 μL of well-mixed whole blood on glass slides. Air-dried smears were fixed in absolute methanol and stained with a Leishman-Giesma solution. The stained smears were cover-slipped using Clarion mounting medium (Sigma-Aldrich, Inc., U.S.A.), and examined and photographed by light microscopy under oil emersion at 400x magnification. For each slide, areas were randomly chosen and 100 leukocytes were counted and differentiated into three different types (lymphocytes, granulocytes, and thrombocytes) based on their morphology (Zinkl et al. 1991; Tavares-Dias 2006). Differential leukocyte concentrations were calculated by multiplying proportional counts by total leukocyte count as measured by flow cytometry. All cell counts were averaged by site and sex.

Ovary Histology

Oocytes were classified as one of four stages based upon levels of vitellogenesis. Stage one follicles exhibited a uniformly stained cytoplasm and a large central nucleus containing numerous small nucleoli around the periphery. Stage two follicles exhibited the development of a chorion and cytoplasm containing small empty vacuoles. Stage three follicles were characterized by the appearance of vitellogenic granules in the cytoplasm and microvilli extensions from the chorion. By stage four, the cytoplasm of the follicles was entirely consumed by large randomly dispersed vitellogenic and empty-appearing vacuoles. Only stage four vitellogenic follicles sectioned through the germinal vesicle were used for measurements. Follicle measurements were obtained using a Zeiss Axiostar plus light microscope under 50x magnification. Length, width, and area measurements were obtained using a PixelLINK 1394 camera (colour) and PixelLINK capture SE software. Slides were broken into three fields of view, and from each field of view four follicles were chosen at random for measurement. Length was measured using the longest dimension of the follicle. Width was then obtained by measuring the follicle.
dimension perpendicular to the midpoint of the length. Each follicle was then outlined with the area perimeter tool which subsequently calculated the surface area of each egg section. Length, width, and area values were respectively averaged between all fields of view for each slide.

In Vitro Ovarian Steroid Production

Determination of in vitro steroid production by fish gonadal tissue was conducted on 10 to 13 female fish per site according to the methods of McMaster et al. (1995). This method has recently been shown to be appropriate for use with common bully ovarian follicles (Landman et al. 2008). Whole ovaries were removed from the fish and sectioned into six roughly equal 20 to 50 mg pieces to be used for basal, forskolin, and human chorionic gonadotropin (hCG) stimulated incubations, each in duplicate. Using 24-well tissue culture plates, individual ovary pieces were placed into separate wells with 500 μL of Medium 199 (Gibco Invitrogen, New Zealand) containing Hank’s salts without bicarbonate supplemented with 25 mM Hepes, 4.0 mM sodium bicarbonate, 0.01% streptomycin sulphate, and 0.1% bovine serum albumin (pH 7.4). Prior to incubation, media was removed and replaced with 500 μL of fresh Medium 199. An additional 500 μL of Medium 199 containing 100 IU/mL hCG (Sigma-Aldrich, St. Louis MO, USA), 10 μM forskolin (SIGMA), or no activator (basal) was added to each well. All treatments were duplicated. Samples were then incubated at 18°C for 24 h, after which the incubation medium was collected by Pasteur pipette, immediately frozen in liquid nitrogen, and stored at -85°C for later steroid determination.

Steroid hormones in incubation media were measured by the standard radioimmunoassay procedure described by McMaster et al. (1992). Frozen incubation media samples were thawed and assayed directly in duplicate on 200-μL aliquots for testosterone (T; Sigma-Aldrich) and 17β-estradiol (E2; Sigma-Aldrich). T and E2 antibodies were obtained from Valeant (Aliso Viejo, Calif., U.S.A). Tritiated T and E2 were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, U.K.). Duplicate steroid determinations were averaged to provide mean steroid production per incubation treatment.

Ethoxyresorufin-O-deethylase (EROD) Activity

Hepatic mixed-function oxygenase enzyme activity was estimated in postmitochondrial supernatant (PMS) as 7-ethoxyresorufin-O-deethylase (EROD) activity, using a modification of the fluorescence plate-reader technique outlined by van den Heuvel et al. (1995). Liver extracts were homogenized in a cryopreservative buffer (0.1 M phosphate, 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol, pH 7.4) and spun at 9,000 g to obtain the PMS. The EROD reaction mixture contained 0.1 M Hepes buffer, pH 7.8 (Sigma, St. Louis, Mo.), 5.0 mM Mg2+, 0.5 mM NADPH (Applichem, Darmstadt, Germany), 1.5 μM 7-ethoxyresorufin (Sigma), and about 0.5 mg/ml PMS protein. The EROD activity was determined kinetically in 96-well plates using one reading every minute for 10 min on a BMG Polarstar Galaxy microplate fluorometer (BMG Labtechnologies, Offenburg, Germany). Resorufin was determined using 544-nm excitation and 590-nm emission filters. Protein content was estimated from fluorescamine fluorescence (390-nm excitation, 460-nm emission filters) against bovine serum albumin standards (Sigma).

Water and Sediment Chemistry

Water and sediment samples were analyzed for organic extractivs according to the methods of Zender et al. (1994) and Tavendale et al. (1995). Frozen effluent samples were thawed and 125 mL of sample was adjusted to pH 9.0 with NaOH. Surrogate standards (2,4,6-tribromoanisole, 2,4,6-tribromophenol, D10-anthracene, D31-palmitic acid, 8(14)-abietenic acid, and dihydrocholesterol) were introduced immediately prior to extraction. Samples were continuously extracted with dichloromethane using glass liquid-liquid extractors. Extracts were passed through sodium sulphate and concentrated with nitrogen using a Zymark Turbovac. The final extract was silylated with bis(trimethylsilyl)-trifluoroacetamide plus 1% trichloromethylsilane and analyzed by gas chromatography with mass selective detection (GC-MSD). All analyte concentrations were corrected for extraction blanks and adjusted for the recovery of the appropriate surrogate standard. Frozen sediment samples were thawed and a 25-mL subsample of the sediment was refrozen at -80°C, freeze-dried for 24 h, and ground to a fine powder using mortar and pestle. An accurately weighed 1-g (±0.001 g) subsample was then homogenized with 9 g of anhydrous granular sodium sulphate (Merck, NZ) using mortar and pestle, and subjected to solid-liquid phase extraction using the same method as for effluent samples.

Statistics

Comparison mean site values for fish mass, liver size, spleen size, and gonad size data were statistically analyzed by analysis of covariance (ANCOVA) on logarithmically transformed variables with body size (length or weight) as the covariate. Male and female data were analyzed separately. Body mass and organ size data are presented in terms of condition and somatic indices for ease of comparison, where: condition factor = (fish mass – visceral mass)/(total length3) × 100; liver somatic index (LSI) = liver mass/(fish mass – visceral mass) × 100; spleen somatic index = spleen mass/(fish mass – visceral mass) × 100; and gonadosomatic index (GSI) = gonad mass/(fish mass – visceral mass) × 100. Ovary mass was excluded from statistical analysis and from GSI calculation when follicles were determined to
be nonvitellogenic by microscopic analysis. Remaining data were compared using analysis of variance (ANOVA) after log-transformation where departures from normality were observed. For data that did not conform to the assumptions of parametric analysis following log-transformation, an equivalent nonparametric test was used. All statistical analyses were performed with STATISTICA v8.0 software (Statsoft, Tulsa, Okla., U.S.A.). The critical level of statistical significance for all tests was $\alpha = 0.05$.

Results

Physiological Indices

No significant differences were observed for weight as it covaries with length (condition factor), or for liver or spleen masses as they covary with body weight (Table 1) between the Tarawera and Rangitaiki bully populations. Gonadal development confirmed both populations were close to the onset of spawning, although the mature Tarawera bullies were significantly more advanced than the Rangitaiki fish, with a mean GSI of 8.27% versus 6.13% for females, and 0.95% versus 0.62% for males, respectively.

Haematology

There were no significant between-site differences found for male common bully haematological parameters (Table 2). Tarawera females possessed slightly smaller erythrocytes (MCV or mean cell volume) with less haemoglobin per cell (MCH or mean cell haemoglobin) compared with Rangitaiki females. Slightly higher mean total leucocyte counts were also observed in Tarawera females. The leucocytes were further differentially classified into three groups based on their morphology (Fig. 2). For both fish populations the lymphocytes were the dominant leucocyte type, while granulocytes and thrombocytes existed in approximately similar proportions. Mean proportional leucocyte counts

| TABLE 1. Mean (±SEM, n) of size and somatic indices in male and female common bully* |
|------------------------------------------|-----------------|-----------------|
| **Males**                                | Rangitaiki      | Tarawera        |
| Length (mm)                              | 88.8 (2.49, 13) | 98.0 (3.86, 19) |
| Weight (g)                               | 10.0 (1.19, 13) | 14.4 (1.78, 19) |
| Condition (K)                            | 1.33 (0.04, 13) | 1.33 (0.04, 19) |
| LSI                                      | 2.19 (0.22, 13) | 2.12 (0.16, 19) |
| GSI                                      | 0.62 (0.10, 13) | 0.95 (0.24, 19)* |
| **Females**                              |                 |                 |
| Length (mm)                              | 95.1 (1.77, 25) | 93.2 (4.02, 20) |
| Weight (g)                               | 12.3 (0.73, 25) | 12.6 (1.64, 20) |
| Condition (K)                            | 1.28 (0.02, 21) | 1.25 (0.06, 14) |
| LSI                                      | 2.46 (0.11, 21) | 3.06 (0.30, 14) |
| GSI                                      | 6.13 (0.48, 21) | 8.27 (1.01, 14)* |

* Asterisks (*) indicate significant difference ($p < 0.05$) in ANOVA between sample sites.

| TABLE 2. Mean (± SEM, n) blood parameters for male and female common bully* |
|------------------------------------------|-----------------|-----------------|
| **Males**                                | Rangitaiki      | Tarawera        |
| Haematocrit (%)                          | 20.0 (1.52, 13) | 18.9 (1.31, 21) |
| Red Blood cells (cells/L × 10^12)        | 1.27 (0.05, 13) | 1.24 (0.08, 21) |
| Mean cell volume (fL)                    | 157 (9.31, 13)  | 151 (4.95, 21)  |
| Haemoglobin concentration (g/L)          | 40.8 (2.33, 13) | 38.7 (2.65, 21) |
| Mean cell haemoglobin (pg/cell)          | 32.2 (1.45, 25) | 32.0 (1.65, 21) |
| Mean cell haemoglobin concentration (g/L)| 215 (16.9, 13)  | 220 (17.2, 21)  |
| White blood cells (cells/L × 10^12)      | 3.78 (0.63, 13) | 3.71 (0.63, 21) |
| Granulocytes (cells/L × 10^9)            | 4.27 (0.61, 12) | 5.46 (0.68, 18) |
| Lymphocytes (cells/L × 10^9)             | 1.70 (0.20, 11) | 1.62 (0.16, 18) |
| Thrombocytes (cells/L × 10^9)            | 8.92 (1.27, 12) | 6.47 (0.83, 18) |
| Spleen somatic index                     | 0.14 (0.02, 12) | 0.14 (0.02, 17) |

| **Females**                              |                 |                 |
| Haematocrit (%)                          | 20.9 (1.09, 25) | 20.0 (1.52, 23) |
| Red Blood cells (cells/L × 10^12)        | 1.25 (0.05, 25) | 1.37 (0.06, 23) |
| Mean cell volume (fL)                    | 169 (6.95, 25)  | 146 (4.50, 23)* |
| Haemoglobin concentration (g/L)          | 40.1 (1.43, 25) | 40.5 (1.92, 23) |
| Mean cell haemoglobin (pg/cell)          | 32.5 (0.82, 25) | 29.7 (0.89, 23)* |
| Mean cell haemoglobin concentration (g/L)| 198 (7.22, 25)  | 207 (7.52, 23)  |
| Leukocytes (cells/L × 10^6)              | 2.80 (0.18, 25) | 3.47 (0.28, 23)* |
| Granulocytes (cells/L × 10^6)            | 8.05 (0.99, 25) | 7.29 (1.40, 16) |
| Lymphocytes (cells/L × 10^6)             | 1.38 (0.09, 25) | 1.98 (0.18, 17)* |
| Thrombocytes (cells/L × 10^6)            | 5.70 (0.43, 24) | 8.95 (0.79, 17)* |
| Spleen somatic index                     | 0.13 (0.01, 18) | 0.13 (0.02, 15) |

* Asterisks (*) indicate significant difference ($p < 0.05$) in ANOVA between site parameters.
were similar between sites, although Tarawera females possessed greater concentrations of both lymphocytes and thrombocytes.

**EROD Activity**

Significant site differences were observed for hepatic EROD activity (Fig. 3). The Tarawera fish demonstrated approximately 6- to 9-fold greater activity compared with the Rangitaiki fish.

![Fig. 2. Representative examples of common bully blood cells in Leishman-Giesma stained blood films viewed at 400× magnification. Arrows indicate leukocytes classified by morphology as granulocytes (1,2), lymphocytes (3), and thrombocytes (clustered, spindle & rods) (4,5,6). All background cells are erythrocytes.](image)

![Fig. 3. Mean hepatic ethoxyresorufin-O-deethylase (EROD) activity (pmol/resorufin/mg/min) in common bully from the Tarawera and Rangitaiki Rivers. Error bars represent SEM; * = p < 0.05.](image)

**Ovary Histology**

The bully ovary was characterized by large vitellogenic eosinophilic oocytes interspersed with small basophilic primary oocytes (less than ten percent of the diameter of the vitellogenic oocytes) (Fig. 4). Ovarian histology revealed that 100% of Rangitaiki River bully were vitellogenic, whereas 76.3% of Tarawera River bully were vitellogenic. No ovulated follicles were present in any of the samples. This pattern was characteristic of the late maturational states of a synchronously spawning species. Sections were examined for atretic follicles and other abnormalities, but none were observed. The mean (SEM, n) vitellogenic follicle diameters were not significantly different at 0.76 (0.03, 25) and 0.80 (0.04, 12) mm for the Rangitaiki and Tarawera sites, respectively. Perimeter and area of the follicles were also evaluated and no significant differences between sites were found (data not shown).

**In Vitro Ovarian Steroid Production**

In general, ovarian follicular steroid production was greatest for Tarawera fish. There were no basal treatment site differences for either sex steroid measured (Fig. 5). Induction of T production by both forskolin and hCG was 2 to 3 times higher in the Tarawera bully follicles compared with Rangitaiki (Fig. 5A). Similarly, the ability of Tarawera bully follicles to produce E2 through activation by forskolin was also slightly higher than the reference fish, although no difference was observed between sites with hCG activation (Fig. 5B).
Wild Common Bully Health in the Tarawera River, NZ

Water and Sediment Chemistry

Organic extractives chemistry of the downstream Tarawera River site reflected the relative inputs into the river system from the pulp and paper mills, showing high variation in chemical components compared with the Rangitaiki River site (Table 3). Both river sites contained detectable amounts of fatty acids and untransformed resin acids. The Tarawera River water and sediment was characteristic of a system receiving pulp and paper mill effluent, having much higher levels of resin acids, namely abietic acid and dehydroabietic acid. Resin acid neutrals were not detected in either river water samples, but low levels were measured in downstream Tarawera River sediment samples. The resin acid neutrals were dominated by retene and tetrahydroretene.

Discussion

In an attempt to establish the suitability of the selected monitoring species and reference population, a number of earlier studies sought to understand the general biology and ecology of wild common bully in the Tarawera and Rangitaiki River systems (van den Heuvel et al. 2007; Michel et al. 2008; Bleackley et al. 2009). The most recent study established that the paired downstream river populations possessed similar reproductive timing and life histories (Bleackley et al. 2009), subsequently enabling this comparative physiological health assessment. Using a broad selection of biological endpoints, this study demonstrated that the Tarawera common bully population were exposed to contaminants capable of inducing hepatic EROD activity in the general absence of other notable physiological effects in comparison with the Rangitaiki River bully population.

In previous studies, considerable effort was focused on determining an appropriate reference population for the Tarawera River bully population. Earlier studies showed that upstream common bully had substantially different reproductive timing than the common bully in the mill effluent receiving environment (van den Heuvel et al. 2007; Michel et al. 2008; Bleackley et al. 2009). Amplified fragment length polymorphism genetic fingerprint analysis showed that upstream, nonmigratory
TABLE 3. Mean concentrations of organic extractives in water and sediment samples from the Tarawera and Rangitaiki River fish sampling sites

<table>
<thead>
<tr>
<th></th>
<th>Water samples (µg/L)</th>
<th>Sediment samples (µg/g dry sediment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rangitaiki</td>
<td>Tarawera</td>
</tr>
<tr>
<td><strong>Phenolics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanilin</td>
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<td>n.d.</td>
</tr>
<tr>
<td><strong>TOTAL PHENOLICS</strong></td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td><strong>Fatty Acids</strong></td>
<td></td>
<td></td>
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<tr>
<td>Decanoic acid</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Dodecanoic acid</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>60.80</td>
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<td>Margaric acid</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Oleic acid</td>
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<td>n.d.</td>
</tr>
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<td>Stearic acid</td>
<td>1.55</td>
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<td>n.d.</td>
</tr>
<tr>
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<td>n.d.</td>
</tr>
<tr>
<td>Tetracosanoic acid</td>
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<td>n.d.</td>
</tr>
<tr>
<td><strong>TOTAL FATTY ACIDS</strong></td>
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<td>10.27</td>
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<tr>
<td><strong>Resin Acid Neutrals</strong></td>
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<td>Fichtelite</td>
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</tr>
<tr>
<td>Dehydroabietin</td>
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</tr>
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<tr>
<td><strong>TOTAL RESIN ACID NEUTRAL</strong></td>
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</tr>
<tr>
<td><strong>Resin Acids</strong></td>
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<td></td>
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<tr>
<td>Pimaric acid</td>
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</tr>
<tr>
<td>Sandaracopimaric acid</td>
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<td>n.d.</td>
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<td>n.d.</td>
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<td>12,14-Dichlorodehydroabietic acid</td>
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<td>n.d.</td>
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<tr>
<td>7-Oxodehydroabietic acid</td>
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<td>n.d.</td>
</tr>
<tr>
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<td>53.67</td>
</tr>
<tr>
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<td></td>
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</tr>
<tr>
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<td><strong>TOTAL PHYTOSTEROLS</strong></td>
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<td>2.29</td>
</tr>
<tr>
<td><strong>TOTAL EXTRACTIVES</strong></td>
<td>68.85</td>
<td>66.22</td>
</tr>
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</table>

*For each compound that was measured above detection for at least one of the four samples analyzed.

^ n.d. = not detected; method detection limit is 0.01 µg/g.
populations of common bully were genetically distinct from the downstream migratory populations (van den Heuvel et al. 2007; Michel et al. 2008). However, the downstream Tarawera population was genetically and reproductively similar to the Rangitiki River population. Hence the use of another river as a reference location (van den Heuvel et al. 2007; Michel et al. 2008; Bleackley et al. 2009). Despite this similarity, individuals representing the genetics of the upstream Tarawera River population were still present downstream, and this is likely why a significant proportion of the downstream bully in the Tarawera River were non-vitellogenic. The differences in reproductive timing would also likely be reflected in other somatic indicators of energy allocation and growth.

Integrated nutritional and/or metabolic stressors are expected to be reflected through changes in energy allocation to somatic growth, as reflected by condition factor (Adams and McLean 1985). Elevated condition is commonly reported for fish inhabiting pulp and paper mill effluent receiving environments as a factor of increased nutrients and water temperature (Munkittrick et al. 1994; Karels et al. 1998). The indicators of growth and energy storage were generally similar between the Tarawera and Rangitaiki populations examined in the current study. Large body size accompanied by generally high condition factor suggests similarly enhanced productivity and/or low population density in both river systems.

Some subtle variation in energy allocation to reproduction was evident based on slightly greater GSI in the Tarawera population. Greater GSI in Tarawera fish was also coupled with increased ovarian steroid biosynthetic capacity. Reproductive disruption is commonly observed in pulp and paper effluent exposed environments, often characterized by reduced circulating sex steroids and gonadal steroid biosynthetic capacity (Munkittrick et al. 1997; Hewitt et al. 2008; Landman et al. 2008). Histological evaluation of ovarian tissue in this study indicated that differences in gonad size were owing to slightly, and presumably natural, advanced reproductive maturity in the Tarawera population. Continued monitoring of reproductive development following this assessment found GSI peaked in Rangitaiki River fish approximately one month later than Tarawera River fish (Bleackley et al. 2009). The absence of any obvious cellular lesions further suggests that differences in reproductive timing were probably not related to any direct toxicant effects.

Although recent rainbow trout (Oncorhynchus mykiss) mesocosm exposures have demonstrated the Tasman Mill effluent may no longer directly induce EROD activity (van den Heuvel and Ellis 2002; van den Heuvel et al. 2008), the current study found significantly elevated EROD activity in wild Tarawera common bully compared with Rangitaiki fish. This observation is somewhat expected given that one of the preliminary wild common bully studies and a 21-d shortfin eel (Anguilla australis) caging study have both demonstrated some degree of EROD induction in situ (van den Heuvel et al. 2006; van den Heuvel et al. 2007). van den Heuvel et al. (2006) speculated that increased EROD activity in caged shortfin eel below the Tasman Mill outfall could potentially be linked to contaminated sediment exposure, such as resin acids and resin acid neutrals. Resin acids that are commonly found in certain pulp and paper mill effluents are known to be transformed in sediments under anaerobic conditions into more toxicologically potent compounds such as polycyclic aromatic hydrocarbons (PAH) (Tavendale et al. 1997a, 1997b). The PAH retene, for example, is known to be bioavailable (Leppanen and Oikari 1999) and capable of inducing EROD activity (e.g., Maria et al. 2005) in some fish species. Similar to the study of van den Heuvel et al. (2006), the current study also found the PAHs retene and tetrahydroretene, albeit in generally low concentrations, in the Tarawera River sediments which were absent in the Rangitaiki River. Contact with these chemicals in the Tarawera is almost certain given the demersal nature of the common bully.

General haematological values were slightly lower than those previously observed for wild common bully (West 2007). In the current study, haematology was relatively consistent between the populations, with the exception of Tarawera females. The lower MCH and MCV values of Tarawera females is confusing in that it is unusual to see changes in these secondary haematological measures without observing corresponding changes in haematocrit, haemoglobin, and/or erythrocyte count. The greater leukocyte numbers (lymphocytes and thrombocytes) in Tarawera females may be suggestive of an immune response, such as infection or disease for example, or alternatively a function of physiological (reproductive) status. These unusual haematological results cannot be adequately explained in this study alone. However, given the relatively small magnitude of the differences, these results are not considered to be of particular concern. Future investigations examining seasonal changes in haematological and immunological parameters may be required to fully understand these observations.

This is the first successful comparative physiological health assessment of wild fish populations in the Tarawera and Rangitaiki Rivers. In line with the disappearance of physiological effects in recent controlled laboratory and mesocosm experiments, this study further demonstrates that, with the exception of EROD induction, characteristic pulp and paper mill effluent effects are not obvious in wild fish in situ. The results of this investigation contribute to a larger body of research aimed at assessing the biotic impacts of effluent discharges to the Tarawera River through tracking ongoing changes and improvements of the combined pulp and paper mill effluent. In addition, the findings of this study can be used in future investigations by acting as a benchmark for comparison.
References


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Impact of a Kraft Pulp and Paper Mill Effluent on Phytoplankton and Macroinvertebrates in River Nzoia, Kenya

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Phytoplankton and macroinvertebrate assemblages were used to assess the impact of a kraft pulp and paper mill effluent in Kenya, on River Nzoia downstream of the discharge point in relation to changes in water quality during May to June and November 2008 (rainy and dry seasons, respectively). Total phosphorus concentration increased from 0.027 mg·L⁻¹ upstream to 0.04 mg·L⁻¹ downstream. Ammonia nitrogen (NH₃-N) concentration was 0.51 mg·L⁻¹ upstream and 0.86 mg·L⁻¹ downstream. Nitrate concentration stood at 1.18 mg·L⁻¹ upstream compared with the 2.23 mg·L⁻¹ downstream. The pH changed from 4.5 to 5.0 upstream to 5.5 to 6.0 downstream, while DO increased from 6.57 to 7.03 mg·L⁻¹ downstream. The BOD₅ (biochemical oxygen demand after five days) values remained almost unchanged from 4.63 mg·L⁻¹ upstream to 4.67 mg·L⁻¹ downstream. Taxon composition of phytoplankton and macroinvertebrates correlated with adverse environmental gradients resulting from the mill's effluent discharge. Overall, there was a shift in composition and abundance of both phytoplankton and macroinvertebrates, with the downstream site recording high numbers of tolerant taxa (i.e., Microcystis sp. and Chironomus sp.). It was recommended that water quality monitoring with effluents of this nature be done using a combination of chemical analysis and biological indicators such as phytoplankton and macroinvertebrates.

Key words: macroinvertebrate, biomonitoring, effluent, nutrient, pulp and paper, pollution

Introduction

Pulp mill effluents have been associated with a number of impact types on water quality and aquatic biota in the receiving water bodies. Several methods have been developed to monitor or assess the impact of paper mill effluents on receiving waters. They include the application of multistable isotope assays (Dubé et al. 2005), chemical analysis, endocrine assessment of fish (McMaster et al. 2005), caging small-bodied fish (Palace et al. 2005), and fathead minnow (Pimephales promelas) life cycle tests (Parrott 2009). Most of these methods are quite expensive and very few are in common practice or remain purely experimental. The use of biotic communities such as diatoms (Eloranta 1995, 1999; Eloranta and Kwandrans 1996; Eloranta and Anderson 1998) and fish (Kovacs et al. 2002; Siligato and Böhmer 2002) to survey gradients of aquatic environment variables has several advantages over physical and chemical monitoring (Kwang-Guk et al. 2002). For instance, in running waters where the water quality changes rapidly, biological monitoring has proved to be a very useful tool due to its integrating nature. While most research on pulp and paper mill effluent has focused on the impact on fish and fisheries resources, studies that have investigated the effects on benthic assemblages have reported an increase in abundance, together with some combination of increases, decreases, or no change in taxon richness, depending on the degree of eutrophication (Sprague and McLeese 1968; Marier 1973; Shumway and Palensky 1973; Culp et al. 2000). Other studies have revealed uptake of contaminants by benthic fauna in areas exposed to pulp and paper mill effluents (Etiegni et al. 2007; Meriläinen and Oikari 2008). Results from these studies show that the evaluation of biotic communities offers a comprehensive alternative to the use of physicochemical parameters when assessing the effect of pulp and paper mill effluents on the aquatic environment.

In Kenya, the impact of pulp and paper mill effluent discharge on River Nzoia has been of major concern over the years (Balirwa and Bugenyi 1988; Achoka 1998). Previous studies have indicated a decrease in fish richness (Balirwa and Bugenyi 1988) and deteriorated water quality (Achoka 1998) downstream of the effluent discharge point. However, because of their mobility, fish cannot give a comprehensive account on the impact of pulp mill effluent as opposed to benthic and/or pelagic assemblages which are more sedentary. The purpose of this study was to investigate the impact of pulp and paper mill effluent on the composition and occurrence of phytoplankton and macroinvertebrate assemblages in relation to changes in water quality arising from discharges from a pulp and paper mill effluent. The hypothesis tested in this study was that the mill effluent has no significant effect on physicochemical parameters and the community structure of phytoplankton and macroinvertebrate assemblages. Such information is necessary in assessing, monitoring, and managing the river and other similar water bodies.

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Materials and Methods

Study Area

The study was conducted at the Webuye PanPaper Mills (Fig. 1a) along River Nzoia. The mouth of River Nzoia lies at latitude 0°3′38.8″N and longitude 33°57′47.33″E. River Nzoia originates from the Cherangani Hills at a mean elevation of 2,300 m above sea level and drains into Lake Victoria at an altitude of 1,000 m above sea level. PanPaper (0°39′54.84″S, 34°47′47.44″E), the only kraft pulp and paper mill in Kenya, has an annual production capacity of 120,000 tonnes of paper from kraft cooking of chips from pine (Pinus patula), cypress (Cupressus lusitanica), and eucalypts (Eucalyptus saligna) and stone grinding of pine (Pinus patula). The bleaching sequences used are CEHH or CEHP (C = chlorination; E = hot caustic extraction; H = hypochlorite application; P = peroxide application) (Dence and Reeve 1996). The paper products include mainly newsprint, writing, and packaging papers for local and export markets in East and Central Africa and Asia (Orori et al. 2005).

PanPaper is situated at an altitude of 1,200 m above sea level on the western part of Kenya (Fig. 1b). The area experiences two rainy seasons. The long rains fall between March and July while the short rains start from August to October. The mean annual rainfall varies from 1,250 to 1,800 mm. The main farming activities in the catchment upstream of the water intake point into the mill include agriculture, agroforestry, forestry, and livestock rearing. The mill consumes about 40,000 m³ of water and discharges between 35,000 to 40,000 m³ daily into the river at a dilution rate varying between 0.3 to 3.2%, depending on the prevailing weather conditions in the area. The mill's effluent takes six weeks to flow through a set of settling tanks (one primary and one secondary), aerated lagoons, and stabilization ponds before being discharged into the river. Over the past 15 years, however, expansion programs within the mill have led to an overloaded wastewater treatment system that was initially designed to treat only 25,000 m³ of mill effluent per day. As a consequence, partially treated mill wastewater is being discharged into River Nzoia in complete violation of the 2006 Effluent Discharge Standards (Table 1). For example, although PanPaper wastewater pH and total dissolved solids (TDS) are in compliance with regards to Kenya's environmental regulations, the mill's treated effluent biochemical oxygen demand after five days (BOD₅) and chemical oxygen demand (COD) are much higher than the stipulated maximum discharge limits of 30 and 50 mg·L⁻¹, respectively. For PanPaper, meeting the 15° H for effluent colour has always been elusive, despite numerous attempts at increasing aeration in the aerated lagoons. More work will probably be required in terms of process modification, higher fibre recovery, and more water recycling for the mill to meet the 2006 Effluent Discharge Standards (Table 1).

Study Site, Sampling, and Sample Preparation

An upstream-downstream design was employed in this study. The two sites were separated by a distance of about 1 km. The downstream site was located 50 m below the discharge point after complete mixing of the effluent and river water that occurs after a short stretch from the outfall due to high turbulence. The two sites share similar environmental attributes including both instream and riparian habitat. The substrates in pools were composed of mud, sand, and detritus while the riffles were made of boulders and pebbles. The fast water flow enhanced mixing and oxygenation. Both sites were being used for drinking water for domestic livestock and sand mining. There was no other discernable point source pollution between the sampling sites besides the discharge of the mill's effluent.

Phytoplankton sampling. Sampling was performed in triplicate at the upstream and downstream sites of the paper mill effluent discharge point on each visit during the months of May, June, and November 2008. Oblique

Fig. 1. Map of the study area showing the location of PanPaper Mills at Webuye in Western Kenya. Map of Kenya at the top left corner (left). Map of Africa showing the location of PanPaper Mills (right).
tows were made with a 28-μ plankton net of 50-cm diameter. The net was cast while standing in the water at a safe depth from the bank. Brisk tows were made so that the mouth of the net was not allowed to touch the river bottom. The pay-length of the towing rope was 2.5 m, allowing an effective towing distance of about 2.0 m, thereby giving an efficiency of about 80%. Samples were collected for phytoplankton analysis according to APHA (1998). In the laboratory, the phytoplankton were identified and counted in a Sedgewick Rafter cell (Lund et al. 1958) using an inverted Olympus CK2 Microscope at ×400, while identification of phytoplankton was done at the mechanical stage of a compound microscope at ×800 to the lowest taxonomic unit using several keys and illustrations (Prescott 1962; Vollenweider 1969; Kramer and Lange-Bertalot 1986, 1988, 1991a, 1991b; APHA 1998).

Phytoplankton density mL⁻¹ (D) = [(A)(l x w x d)] (1)

where $D$ = phytoplankton density for subsample of mL⁻¹ in number; $A$ = average number of phytoplankton counted in one Sedgewick-Rafter cell; $l$ = length in mm of the Sedgewick Rafter counting cell (50); $w$ = width of the Sedgewick Rafter counting cell (20 mm); $d$ = depth of the Sedgewick Rafter counting cell (1 mm).

The counted number of phytoplankton cells in each 1-mL subsample was converted to the original 50-mL sample by the following relationship:

\[ \text{Phytoplankton density in 50 mL (T)} = D \times V_1 \] (2)

where $D$ = phytoplankton density for subsample in numbers per unit volume (mL⁻¹); $V_1$ = volume of the original sample (50 mL). The 50-mL volume was converted to the total volume filtered during the oblique tows by the following relationship:

\[ \text{Final phytoplankton density} = T \times (1000/V_2) \] (3)

where $T$ = phytoplankton density for 50-mL sample; $V_2$ = original volume filtered during the net tows;

Sample volume ($V_2$) = $\pi r^2 d$ (4)

where $\pi = \pi$, with a value of 3.14; $r$ = the radius of the plankton net mouth (25 cm); $d$ = distances moved by the net during towing (200 cm).

The average distance towed was determined from the pay-out tow rope length of 2.5 m resulting in an effective towing distance of 2.0 m (200 cm), and all tows were done while standing in water on the shallow edge of the stream at each sampling site.

**Water quality sampling.** Water samples were collected in triplicate once in May to June and November 2008 for nutrient analysis following standard procedures (APHA 1998). The samples were packed in fresh ice in a cooler box then transported to the laboratory for analysis of nitrate nitrogen (NO₃⁻N), ammonia nitrogen (NH₄⁻N), and total phosphorus (TP). Water temperature and dissolved oxygen (DO) were determined in situ at each of the sampling sites using a JENWAY 3405 electrochemical

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**TABLE 1. Raw and treated kraft pulp and paper mill effluent by current treatment system**

<table>
<thead>
<tr>
<th>Parameters *</th>
<th>Primary clarifier overflow</th>
<th>Treated after last stabilization lagoon</th>
<th>Effluent discharge standards b</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.5–9.3</td>
<td>6.9–7.5</td>
<td>6.5–8.5 (nonmarine)</td>
</tr>
<tr>
<td>Alkalinity (mg/L)</td>
<td>330.0–346.4</td>
<td>70.0–88.2</td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>39.0–39.6</td>
<td>19.0–21.2</td>
<td></td>
</tr>
<tr>
<td>TS (mg/L)</td>
<td>872.4–980.7</td>
<td>440.0–474.5</td>
<td>30.0</td>
</tr>
<tr>
<td>TDS (mg/L)</td>
<td>670.0–699.6</td>
<td>670.0–699.6</td>
<td>1,200.0</td>
</tr>
<tr>
<td>TSS (mg/L)</td>
<td>212.5–291.5</td>
<td>94.6–133.0</td>
<td>30.0</td>
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<tr>
<td>Colour (°F)</td>
<td>1,280.5–1,867.7</td>
<td>1,600.0–3,263.3</td>
<td>15.0</td>
</tr>
<tr>
<td>BOD₅ (mg/L)</td>
<td>182.5–234.7</td>
<td>62.8–117.6</td>
<td>30.0</td>
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<tr>
<td>COD (mg/L)</td>
<td>536.0–591.5</td>
<td>296.7–401.5</td>
<td>50.0</td>
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<tr>
<td>Turbidity (NTU)</td>
<td>130.0–136.1</td>
<td>311.0–351.3</td>
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<tr>
<td>Conductivity (μS/cm)</td>
<td>1,339.2–2,109.3</td>
<td>790.0–891.3</td>
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<tr>
<td>Dissolved oxygen (mg/L)</td>
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<tr>
<td>Phosphorus (mg/L)</td>
<td>0.056</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Nitrites (mg/L)</td>
<td>0.004</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>Nitrate (mg/L)</td>
<td>0.02</td>
<td>0.036</td>
<td></td>
</tr>
</tbody>
</table>

*TS = total solids; TDS = total dissolved solids; TSS = total suspended solids; BOD₅ = biochemical oxygen demand after 5 d; COD = chemical oxygen demand.

Determination of nitrate. Nitrate concentration was determined spectrophotometrically after nesslerization using NEDD solution at a wavelength of 543 nm; nitrite-nitrogen (NO2-N) concentrations were obtained from a standard calibration graph and converted into nitrate by multiplication with a factor of 3.28 as follows:

$$1 \text{ mg}\cdot\text{L}^{-1} \text{NO}_2\text{-N} (46.01 \text{ mg NO}_2/14.01 \text{ mg N}) = 3.28$$  \hspace{1cm} (5)

Then $\text{NO}_3 = 3.28$ (concentration of NO2-N from the calibration graph).

Determination of ammonia. Ammonia nitrogen was measured as total Kjeldahl nitrogen. The digest was transferred to a micro Kjeldahl distillate unit to which about 3.5 mL of hypo solution was added. Five (5) mL of baric acid solution containing 3 to 4 drops of indicator was put into a conical flask. The boiling flask was heated to pass the steam into the sample; distillation was continued for about 10 minutes. The conical flask was then removed and the boiling flask cooled so that all wastes were sucked and removed through a tap. The distillate was titrated against hydrochloric acid; turning from blue colour to pink was the end point.

Determination of phosphate. Phosphate concentration was determined by the spectrophotometric method. The absorbance and standard against a reagent blank was read at a wavelength of 882 nm. Phosphate concentration ($\text{PO}_4^{3--}\text{P}$) in mg·L$^{-1}$ was given as:

$$F = \text{standard solution (}\mu\text{g PO}_4^{3--}\text{P})/(E_s \text{ standard } - \text{EB}_s)$$  \hspace{1cm} (6)

$$\mu\text{g PO}_4^{3--}\text{P} = F [E_s \text{ sample } - (E_0 + \text{EB}_s)]$$  \hspace{1cm} (7)

where $E_0$ = absorbance of sample without reductant; $E_s$ = absorbance of standard or standard solution without reductant; $\text{EB}_s$ = absorbance of distilled water + reagent.

Macroinvertebrate sampling. Sampling for macroinvertebrates was carried out where water samples had been collected using a scoop net (0.5-mm mesh size). Triplicate samples were collected by a kick method from runs, riffles, and pools from each site. Sampling was done for a standard three minutes by disturbing a 1-m$^2$ area for each microhabitat (upstream and downstream at depths of less than 0.5, 0.70, and 1.0 m from the river bank (a total of three microhabitats per site). Specimens were sorted live in white plastic trays, then poured into vials and preserved with 70% ethyl alcohol. Having determined that replications in a site did not exhibit any statistical difference, these replicates were pooled to make one composite sample per site. At the laboratory, samples were sieved using a 300-μm mesh size sieve and sorted further. Specimens were identified down to the genus level according to Merritt and Cummins (1996) and Gerber and Gabriel (2002).

Data Analysis

All the data collected were put in a spreadsheet to facilitate statistical analyses using MINITAB Ver. 13 (Minitab Inc. Corporation 2000) statistical package. Data on physicochemical parameters were tested for significant difference between sites and months using the general linear model (GLM) analysis of variance (ANOVA). Data on phytoplankton and macroinvertebrate abundance were also tested for significant difference between sites, months, replicates, and species using GLM ANOVA. This approach allowed for the use of the Student Newman Keules (SNK) multiple range test if significant differences were detected among the categories in the model. Whenever possible, Least Significant Difference (LSD) tests were used to separate the means. The relative abundance of various macroinvertebrate genera were determined to provide information on the make-up of the assemblage and the relative contribution of the macroinvertebrate populations to the total assemblage according to the following formula:

$$\text{Relative abundance} = \frac{\text{Number of individuals of one taxon}}{\text{Total number of individuals}}$$  \hspace{1cm} (8)

Upstream and downstream physicochemical parameters and phytoplankton densities were compared using paired t-tests (Zar 2001). The interrelationships between physicochemical parameters, phytoplankton, and macroinvertebrate assemblages were examined using canonical correspondence analysis (CCA) (Braak 1986; Braak and Prentice 1988; Braak and Verdonschot 1995). CCA is a multivariate direct gradient method designed to extract synthetic environmental gradients from ecological datasets. CCA assumes that species have unimodal distributions along environmental gradients (Minchin 1987; Braak and Smilauer 1998). CCA is calculated using a reciprocal averaging form of correspondence analysis. However, at each cycle of the averaging process, a multiple regression of the sample scores is performed on the environmental variables. New site scores are calculated based on this regression, and then the process is repeated until the scores stabilize. The result is that the axes of the final ordination, rather than simply reflecting dimensions of the greatest variability in the species data, are restricted to the linear combinations of the environmental variables (physicochemical parameters) and the species data (phytoplankton or
Impact of a Kraft Mill Effluent on Aquatic Biota

macroinvertebrate abundance) (Wilson and Mohler 1983; Palmer 1994). In this way these two sets of data are directly related, hence the popularity of this method whose main strength is its robustness to many data types, nonlinear relationships, and some rare species. CCA weakness however remains associated with multiple regression and chi-square distances that can emphasize rare species.

Data on phytoplankton and environmental variables, except pH, were log transformed because of their skewed distributions. Relative abundance values of the macroinvertebrate taxa were arcsine transformed. It is known from statistical theory that percentages or proportions form a binomial rather than a normal distribution, with the deviation from normality being great for small (0 to 30%) or large (70 to 100%) percentages. But if the square root of each proportion is transformed to its arcsine (i.e., the angle whose arcsine is), then the resultant data will have an underlying distribution that is nearly normal. Since count data of macroinvertebrates would follow a binomial distribution, transformation using arcsine and expressing the relative changes of abundance in percentages was appropriate in this study. All statistical tests were carried out at $\alpha = 0.05$.

Results

Physicochemical Water Parameters

The results for treated effluent shown in Table 1 underscore the lack of adequate treatment of the mill’s effluent since its expansion programs. There was a marked increase in temperature, BOD$_5$, nitrites, phosphates, and electrical conductivity downstream as compared with upstream samples. For example, BOD$_5$ and COD were well above the current Effluent Discharge Standards. Only effluent TDS, temperature, and pH appeared to fall within the acceptable limits. For the river water, there was a significant increase in DO (mg·L$^{-1}$) from 6.57 upstream to 7.03 downstream ($p = 0.024$) and nitrate nitrogen (mg·L$^{-1}$) from 1.18 to 2.23 ($p = 0.008$), BOD$_5$ (mg·L$^{-1}$) from 4.63 to 4.67 ($p = 0.032$), TP (mg·L$^{-1}$) from 0.03 to 0.04 ($p = 0.045$), and a threefold increase in electrical conductivity (mS·cm$^{-1}$) from 110 upstream to 333 downstream (Table 2). There was an apparent increase in temperature (ºC) from 21.7 upstream to 23.0 downstream ($p = 0.383$) and ammonia nitrogen from 0.51 to 0.86 (mg·L$^{-1}$) ($p = 0.108$), but these were not statistically significant (Table 2).

Species Composition and Abundance

Data on phytoplankton genera did not show any significant difference between months, replicates, and sites (upstream and downstream). However, there was a significant difference in abundance among species. A total of 36 different genera, belonging to five classes were recorded: Bacillariophyceae, Cyanophyceae, Euglenophyceae, Chlorophyceae, and Pyrrophyceae. The Chlorophyceae were the most numerically abundant and they also had the highest number of genera (15), followed by Bacillariophyceae with 10 genera (Fig. 2). The most abundant genera in Chlorophyceae included Botryococcus, Cyanarcus, Scenedesmus, Botrydium, and Monoraphidium, while the most common and abundant genera in Bacillariophyceae were Melosira, Synedra, Navicula, and Nitzchia. The Cyanophyceae had 4 genera consisting of Microcystis, Synechococcus, and Coenococcus (Fig. 3a). Both Euglenophyceae and Pyrrophyceae had three genera each dominated by Trachelomonas and Netrium, respectively (Fig. 3b and c).

There was a significant difference in phytoplankton abundance between genera ($F_{0.05(2),34,1} = 3.35; p < 0.0005$) but not between sites ($F_{0.05(2),1,174} = 0.29; p = 0.858$), although the upstream site exhibited an apparent but not statistically significant high density. The GLM ANOVA showed significant differences in the observed abundance of Bacillariophyceae ($F_{0.05(2),9,1} = 5.43; p < 0.0005$) and Chlorophyceae ($F_{0.05(2),14,1} = 2.59; p = 0.004$), but not between sites: ($F_{0.05(2),1,49} = 0.26; p = 0.613$) and ($F_{0.05(2),1,74} = 0.25; p = 0.617$), respectively (Fig. 2). The same was

<table>
<thead>
<tr>
<th>TABLE 2. Paired t-test showing mean physicochemical parameters upstream and downstream during May to June and November 2008 a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physicochemical parameter</strong> b</td>
</tr>
<tr>
<td>Temp (ºC)</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>DO (mg/L)</td>
</tr>
<tr>
<td>BOD$_5$ (mg/L)</td>
</tr>
<tr>
<td>NO$_3$-N (mg/L)</td>
</tr>
<tr>
<td>NH$_4$-N (mg/L)</td>
</tr>
<tr>
<td>TP (mg/L)</td>
</tr>
<tr>
<td>Cond. (µS/cm)</td>
</tr>
</tbody>
</table>

a $n = 9$ for all parameter for all months.
bTemp = temperature; DO = dissolved oxygen; BOD$_5$ = biochemical oxygen demand after 5 d; NO$_3$-N = nitrate nitrogen; NH$_4$-N = ammonia nitrogen; TP = Total phosphorus; Cond. = conductivity.
cAsterisk (*) indicates significant differences.
observed for Cyanophyceae ($F_{0.05(2),3,1} = 3.31; p = 0.042$) and Euglenophyceae ($F_{0.05(2),1} = 0.91; p = 0.423$), but not between sites: ($F_{0.05(2),1,19} = 0.22; p = 0.642$) and ($F_{0.05(2),1,14} = 0.03; p = 0.872$), respectively (Fig. 3a,b,c). Further analysis using multiple range tests identified the gradient of abundance between the two sites and facilitated the determination of possible explanations based on physicochemical parameters. It was then possible to identify the sensitive, tolerant, and intolerant groups of phytoplankton in the samples (Table 3).

The SNK multiple range test and LSD indicated that for Bacillariophyceae, *Melosira* was significantly more abundant at the two sites than all the other genera ($p = 0.004$), possibly indicating that it is tolerant to the pulp and paper mill effluents. *Synedra* and *Eunotia* abundance increased by almost a similar proportion of 100% from upstream to downstream sites (Table 3). Other genera such as *Rhoicosphenia*, *Gomphocymbella*, and *Cocconeis*, which were absent upstream, suddenly appeared downstream in relatively high abundance, indicating their sensitivity in determining the impact of pollutants from the pulp and paper effluent (Fig. 2a). *Synechococcus* increased twofold downstream. For the Chlorophyceae the abundance of *Botryococcus* downstream was significantly higher than all the other genera at the two sites, thereby showing tolerance to the effluents (Fig. 2b). However, *Botrydyium* density was significantly lower than that of *Botryococcus* ($p < 0.0005$) but not for any other genera. *Botrydium*

**Fig. 2.** Variation in phytoplankton genera of Bacillariophycea (a) and Chlorophyceae (b) for two sampling stations above the intake and below the discharge into the river. (Horizontal bars are standard errors).
Fig. 3. Variation in phytoplankton genera of Cyanophyceae (a), Euglenophyceae (b), and Pyrrophyceae (c) for two sampling stations above the intake and below the discharge into River Nzoia and environmental gradients (horizontal bars are standard errors).
could be considered as a sensitive species that disappeared with deteriorating water quality downstream (Table 3). The abundance of *Botryococcus*, *Scenedesmus*, and *Chlamydomonas* had significantly increased (by 37, 154, and 100%, respectively) downstream compared with their abundance upstream (Fig. 2b; Table 3). These genera also are indicators of the deteriorating water quality. The Pyrrophyceae genus *Closteridium* was completely absent downstream (Fig. 3c; Table 2). For Euglenophyceae, the genus *Phacus* also appeared only in the upstream station but not downstream (Fig. 3b). For Euglenophyceae, the genus *Phacus* also appeared only in the upstream station but not downstream (Fig. 3b; Table 3). These genera are also considered to be sensitive (Table 3). The relative abundance of macroinvertebrate taxa was thus compared only between upstream and downstream sites. Macroinvertebrate samples at the upstream site were dominated by Ephemeroptera taxa which together with Plecoptera and Trichoptera (EPT) formed more than 45% of the total number of individuals collected from the site (Table 4). At the downstream site the abundance of EPT was reduced while that of Diptera increased, constituting more than 58% of the total number of individuals sampled. There was a reduction in the number of taxa from 18 at the upstream site to 15 at the downstream site where most of the Ephemeroptera taxa were replaced by the Diptera (Table 5).

**Canonical Correspondence Analysis (CCA)**

Based on the t-test performed on the river physicochemical parameters, it was observed that the variation in populations of certain phytoplankton genera coupled with changes in river water quality were illustrated using the multivariate approach, CCA.

**Bacillariophyceae.** The CCA triplot showed that Bacillaria and *Navicula* were associated with high

<table>
<thead>
<tr>
<th>Effects</th>
<th>Genera</th>
<th>Percent change</th>
<th>Implication</th>
<th>Reference</th>
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<tr>
<td>Loss</td>
<td><em>Bacillaria</em></td>
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<td>this study</td>
</tr>
<tr>
<td></td>
<td><em>Botrydium</em></td>
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<td>sensitive</td>
<td>this study</td>
</tr>
<tr>
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<td><em>Gastrophora</em></td>
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<td>this study</td>
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<td>this study</td>
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<td><em>Cyamarcus</em></td>
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<td><em>Pinularia</em></td>
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<td>sensitive</td>
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<td>Appearance</td>
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<td>pollution indicator</td>
<td>Unni and Pawar 2000</td>
</tr>
<tr>
<td></td>
<td><em>Stigeoclonium</em></td>
<td>100</td>
<td>tolerant to nutrients</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td><em>Cocconeis</em></td>
<td>100</td>
<td>tolerant to nutrients</td>
<td>Lung’ayiah et al. 2000</td>
</tr>
<tr>
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<td><em>Botryococcus</em></td>
<td>89</td>
<td>tolerant to nutrients</td>
<td>Soininen 2002</td>
</tr>
<tr>
<td></td>
<td><em>Chlamydomonas</em></td>
<td>100</td>
<td>tolerant to nutrients</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td><em>Synechococcus</em></td>
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<td>tolerant to nutrients</td>
<td>Akbay et al. 1999</td>
</tr>
<tr>
<td></td>
<td><em>Melosira</em></td>
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<td>Lung’ayiah et al. 2000</td>
</tr>
<tr>
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<td><em>Eunotia</em></td>
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<td>tolerant to nutrients</td>
<td>Soininen 2002</td>
</tr>
<tr>
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<td><em>Netrium</em></td>
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<td>this study</td>
</tr>
<tr>
<td></td>
<td><em>Scenedesmus</em></td>
<td>133</td>
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<td>Unni and Pawar 2000</td>
</tr>
<tr>
<td></td>
<td><em>Spirulina</em></td>
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<td>tolerant to nutrients</td>
<td>Okoth et al. 2009</td>
</tr>
<tr>
<td></td>
<td><em>Synedra</em></td>
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<td>tolerant to nutrients</td>
<td>Akbay et al. 1999</td>
</tr>
<tr>
<td></td>
<td><em>Trachelomonas</em></td>
<td>1,800</td>
<td>tolerant to nutrients</td>
<td>this study</td>
</tr>
<tr>
<td>Decrease</td>
<td><em>Euglena</em></td>
<td>50</td>
<td>sensitive</td>
<td>Arimoro et al. 2008</td>
</tr>
<tr>
<td></td>
<td><em>Microcystis</em></td>
<td>57</td>
<td>tolerant to nutrients</td>
<td>Akbay et al. 1999</td>
</tr>
</tbody>
</table>
TABLE 4. The distribution and relative abundance (arc sine numbers) of macroinvertebrate taxa upstream and downstream in the River Nzoia during May-June and November 2008\footnote*{n = 9 for all months.}

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Upstream</th>
<th>Downstream</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ephemeroptera</td>
<td>Baetidae</td>
<td>Baetis</td>
<td>23.1</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Baetidae</td>
<td>Centropilum</td>
<td>3.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Caenidae</td>
<td>Caenis</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Ecdyonurida e</td>
<td>Ecdyonurus</td>
<td>3.1</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>Heptageniidae</td>
<td>Heptagenia</td>
<td>6.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Ecdyonuridae</td>
<td>Rithrogena</td>
<td>4.6</td>
<td>-</td>
</tr>
<tr>
<td>Plecoptera</td>
<td>Peridiae</td>
<td>Dinocera</td>
<td>1.5</td>
<td>4.2</td>
</tr>
<tr>
<td>Trichoptera</td>
<td>Leptoceridae</td>
<td>Athropassae</td>
<td>-</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>Philopotamidae</td>
<td>Philopotamus</td>
<td>3.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Amphipoda</td>
<td>Branchyura</td>
<td>Caecinus</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>Coleoptera</td>
<td>Dytiscidae</td>
<td>Platambus</td>
<td>9.2</td>
<td></td>
</tr>
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<td></td>
<td>Elmidae</td>
<td>Elmis</td>
<td>4.6</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>Gyriidae</td>
<td>Gyrius</td>
<td>13.9</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Hydremniidae</td>
<td>Hydremnus</td>
<td>-</td>
<td>1.6</td>
</tr>
<tr>
<td>Diptera</td>
<td>Chironomidae</td>
<td>Chironomus</td>
<td>-</td>
<td>36.5</td>
</tr>
<tr>
<td></td>
<td>Simulidae</td>
<td>Simulium</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Tabanidae</td>
<td>Tabanus</td>
<td>-</td>
<td>19.8</td>
</tr>
<tr>
<td></td>
<td>Tipulidae</td>
<td>Pedicia</td>
<td>-</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eliptera</td>
<td>3.1</td>
<td></td>
</tr>
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<td>Hemiptera</td>
<td>Belostomatidae</td>
<td>Belostoma</td>
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<td>4.2</td>
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<tr>
<td></td>
<td>Gerridae</td>
<td>Gerris</td>
<td>7.7</td>
<td>-</td>
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<tr>
<td></td>
<td>Velidae</td>
<td>Vella</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>Lamellibranchiata</td>
<td>Sphaeridae</td>
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<td>-</td>
<td></td>
</tr>
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<td>Odonata</td>
<td>Agridae</td>
<td>Agrion</td>
<td>3.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cordulidae</td>
<td>Epiconela</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

TABLE 5. Upstream to downstream benthic macroinvertebrate genera comparisons; loss and appearance of genera as well as significant increase or decrease in the abundance of a genera are indicated

<table>
<thead>
<tr>
<th>Effect</th>
<th>Genera</th>
<th>Percent change</th>
<th>Pollution tolerance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss</td>
<td>Centropilum</td>
<td>100 Sensitive</td>
<td>Polluted tolerant</td>
<td>Barbour et al. 1999</td>
</tr>
<tr>
<td></td>
<td>Heptagenia</td>
<td>100 Sensitive</td>
<td>Polluted tolerant</td>
<td>Raburu et al. 2009</td>
</tr>
<tr>
<td></td>
<td>Rithrogena</td>
<td>100 Sensitive</td>
<td>Polluted tolerant</td>
<td>Raburu et al. 2009</td>
</tr>
<tr>
<td></td>
<td>Platambus</td>
<td>100 Sensitive</td>
<td>Polluted tolerant</td>
<td>Barbour et al. 1999</td>
</tr>
<tr>
<td></td>
<td>Eliptera</td>
<td>100 Sensitive</td>
<td>Moderately tolerant</td>
<td>Barbour et al. 1999</td>
</tr>
<tr>
<td></td>
<td>Gerris</td>
<td>100 Moderately tolerant</td>
<td>Barbour et al. 1999</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agrion</td>
<td>100 Moderately tolerant</td>
<td>Barbour et al. 1999</td>
<td></td>
</tr>
<tr>
<td>Appearance</td>
<td>Hydremnus</td>
<td>100 Polluted tolerant</td>
<td>Barbour et al. 1999</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chironomus</td>
<td>100 Polluted tolerant</td>
<td>Barbour et al. 1999</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Simulium</td>
<td>100 Moderately tolerant</td>
<td>Masese et al. 2009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tabanus</td>
<td>100 Polluted tolerant</td>
<td>Barbour et al. 1999</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arthropassae</td>
<td>100 Polluted tolerant</td>
<td>Barbour et al. 1999</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nycptiphanes</td>
<td>100 Polluted tolerant</td>
<td>Barbour et al. 1999</td>
<td></td>
</tr>
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<td></td>
<td>Pedicia</td>
<td>100 Polluted tolerant</td>
<td>Barbour et al. 1999</td>
<td></td>
</tr>
<tr>
<td>Increase</td>
<td>Ecdyonurus</td>
<td>197 Sensitive</td>
<td>Barbour et al. 1999</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dinocera</td>
<td>180 Sensitive</td>
<td>Barbour et al. 1999</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Elmis</td>
<td>59 Moderately tolerant</td>
<td>Buss et al. 2002</td>
<td></td>
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<tr>
<td>Decrease</td>
<td>Baetis</td>
<td>80 Moderately tolerant</td>
<td>Thorne and Williams</td>
<td></td>
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<td></td>
<td>Philopotamus</td>
<td>32 Moderately tolerant</td>
<td>Thorne and Williams</td>
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<td></td>
<td>Gyrius</td>
<td>85 Moderately tolerant</td>
<td>Barbour et al. 1999</td>
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</table>
loading of nitrates on the first CCA axis (Fig. 4), whereas *Gomphocymbella* and *Synedra* were affected by high loading of phosphorus. Negative loading of BOD$_5$ and ammonia was associated with *Nitzchia* at both upstream and downstream sampling sites. *Pinnularia* was however not associated with any factor loading, but showed negative abundance on the first axis upstream.

**Chlorophyceae.** DO and ammonia had positive factor loading on the first axis but negative loading on the second axis (Fig. 5). On the first axis, the factor loading at the upstream site was correlated with high abundance of *Monoraphidium*, *Hydrodactyon*, *Cladophora*, *Botrydium*, and *Haematococcus*. Negative factor loading for nitrates and phosphorus on the first axis at the upstream site was associated with high densities of *Palmelloccocus* and *Nitella*. *Chaetophora* was closely associated with the upstream site without any significant factor loading on the first axis, but with high negative factor loading for DO, BOD$_5$ and ammonia.

**Cyanophyceae.** There was high positive factor loading for nitrates on the first axis at the upstream station for *Synechococcus* and at the downstream site for *Microcystis* (Fig. 6). High factor loading of phosphorus and temperature in the downstream promoted high *Coenococcus* density.

**Euglenophyceae and Pyrrophyceae.** The Euglenophyceae and Pyrrophyceae were represented by only three genera each. There was a positive factor loading of phosphorus, nitrites, and temperature on the first axis downstream (Fig. 7). *Closterium* was closely associated with nitrates while *Netrium* was associated with temperature factor loading downstream. High negative factor loading of ammonia, DO, and BOD$_5$ upstream were associated with high densities of *Phacus* and *Closeridium* on both axes (Fig. 7).

**Macroinvertebrates.** The downstream site was associated with increased pH, BOD$_5$, and conductivity while the upstream site recorded higher DO values (Fig. 8). The CCA grouped taxa into three clusters: those associated with the downstream site and higher pH, BOD$_5$, and conductivity values (*Chironomus* sp., *Tabanus* sp., *Limnius* sp., *Gyrinus* sp., and *Pedicia* sp.), and those associated with the upstream site and higher DO values (e.g., *Heptagenia*, *Agrio* sp., *Gerris* sp., and *Rhithrogena* sp.). The third group displayed no particular preference for either of the two sites (*Ecdyonurus* sp., *Belostoma* sp., and *Philopotamus* sp.). As a general observation, Fig. 2 and 3 show that there was a reduction of taxon richness as one moved from upstream to the downstream sites.

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**Fig. 4.** Canonical correspondence analysis (CCA) ordination plot for the distribution of Bacillariophyceae genera above the intake and below the discharge into River Nzoia and environmental gradients.
Impact of a Kraft Mill Effluent on Aquatic Biota

Fig. 5. Canonical correspondence analysis (CCA) ordination plot for the distribution of Chlorophyceae genera above the intake and below the discharge into River Nzoia and environmental gradients.

Fig. 6. Canonical correspondence analysis (CCA) ordination plot for the distribution of Cyanophyceae genera above the intake and below the discharge into River Nzoia and environmental gradients.
Kramer and Lange-Bertalot (1986) have grouped diatoms into three categories based on their sensitivity to pollution: highly pollution tolerant, moderately pollution tolerant, and pollution-sensitive species. In this study, among the Bacillariophyceae, *Navicula* (which is known to be sensitive and hence a pollution indicator [Soininen 2002]) disappeared from the downstream site (Table 3). *Nitzchia* (Bacillariophyta) *Hydrodacyon, Monoraphidium,* and *Pediastrum* (Chlorophyta) (which are known to be cosmopolitan and insensitive to environmental change) (Lung’ayiah et al. 2000) displayed no particular site preference, with some slight increase downstream. However, nutrient tolerant groups such as *Closterium, Coenococcus, Stigeoclonium* (Arimoro et al. 2008), and *Cocconeis* (Lung’ayiah et al. 2000) were found only in the downstream site (Table 2). Similarly, two species that are known to be indicators of pollution, *Gomphonema* (Lung’ayiah et al. 2000) and *Rhoicosphenia* (Unni and Pawar 2000), showed increased abundance downstream. The increase in density in the downstream site of most of these pollution tolerant taxa was probably associated with high loadings of both nitrates and phosphorus.

Even though there was a decline in *Microcystis* and *Synechococcus* abundance downstream, these species are known to be tolerant to high levels of nutrients (Akbay et al. 1999). This shows that the level of nutrient (nitrogen and phosphorus) in the river from the upper catchment was already high. The high nitrogen and phosphorus was likely the result of intensive application of N:P fertilizers for wheat and maize farming within the upper catchment area (Osano et al. 2003; Raburu et al. 2009). High abundance of *Rhoicosphenia* and *Gomphocymbella,* believed to be good indicators of pollution (Lung’ayiah et al. 2000; Unni and Pawar 2000), were also found downstream probably as a result of increased phosphorus and high temperature (Table 3). High phosphorus in the river at the downstream site may probably result from the incomplete consumption of the diammonium phosphate (DAP) applied by PanPaper to assist the biodegradation of organic matters in its effluent.

High levels of ammonia (0.86 mg·L⁻¹), nitrates (2.23 mg·L⁻¹), and phosphorus (0.04 mg·L⁻¹) favoured the survival and multiplication of *Scenedesmus* and *Pediastrum* downstream as compared with upstream. However, lower loadings of the same factors on a secondary axis favoured *Palmellococcus* and *Nitella* upstream, which are pollution sensitive (Lung’ayiah et al. 2000). High DO levels downstream can be attributed to a high reaeration rate of the river water at the point of effluent discharge because of the high turbulence maintained at this point by design to ensure good mixing and dilution of the mill effluent. However, the impact of the mill nutrients (e.g., deteriorating physicochemical parameters) was still reflected in the biotic communities of phytoplankton and macroinvertebrates. *Stigeoclonium* and *Monoraphidium* appeared to be insensitive to

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**Fig. 7.** Canonical correspondence analysis (CCA) ordination plot for the distribution of Euglenophyta and Pyrrophyceae genera above the intake and below the discharge into River Nzoia and environmental gradients.
Impact of a Kraft Mill Effluent on Aquatic Biota

Changes in the river water quality, while *Botryococcus*, *Scenedesmus*, and *Chlamydomonas* seemed to thrive with an increase of 37, 154, and 100%, respectively, in the face of deteriorating water qualities (Table 5). The cumulative percentage eigenvalues of the CCA explained up to 60% density variation for Bacillariophyta, 70% for Chlorophyta, 95% for Cyanophyta, 77% for Euglenophyta, 85% for Pyrrophyta, and 90% density variation for the macroinvertebrates (Y variables) in relation to the water quality parameters (X variables). The results from this study show that multivariate analysis can be used to link the effects of environmental gradients to phytoplankton and macroinvertebrate assemblages with good analytical results (Miesch 2005). Even though there is need for additional water quality parameters to be determined, only a few of these parameters can be effectively used to explain changes in community structures, and this study has provided a good example of that based on the high cumulative eigenvalues of at least 60%.

High densities of *Microcystis* were correlated with high levels of nitrates, while the densities of *Coenococcus* were correlated with high phosphorus load (Fig. 6). This means that the growth of some genera of Bacillariophyceae, Chlorophyceae, and Cyanophyceae was favoured by nitrate, while others were helped by phosphates and to some extent elevated temperatures. Low levels of ammonia and BOD, upstream seemed to favour *Closteridium* and *Phacus*, which were completely absent downstream (Fig. 7). Increases in temperature and DO resulted in the appearance of *Closterium*, and an increase in both *Netrium* (100%) and *Trachelomonas* (160%) numbers at the downstream site.

The composition of macroinvertebrates at the downstream site was different from that at the upstream site, indicating a probable direct effect of the mill’s discharges on the composition of macroinvertebrate assemblages. The taxa recorded at the upstream site mainly belonged to the pollution sensitive Ephemeroperta (Raburu et al. 2009). As a consequence of the effect of

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Fig. 8. Canonical correspondence analysis (CCA) ordination plot for the macroinvertebrate taxa and environmental gradients at the two sites showing clear separation of sites in the primary axis. The dashed circles encompass taxa associated with environmental conditions peculiar to the two sites.
the effluent discharges on water quality, the sensitive taxa were replaced by tolerant *Chironomus* sp., *Pedicia* sp., *Tabanus* sp. (Diptera), which have been found to be tolerant to high organic loads from industrial discharges (Raburu and Tonderskii 2004).

It can be stated that the impact of effluents from PanPaper Mills on water quality was likely responsible for the reduction in taxon richness at the downstream site. This is based on the fact that the macroinvertebrate taxa recorded at the two sites were low for a sixth-order river, indicating an overall degraded water quality in the river. A relatively unperturbed upstream tributary of the same river had been found to have more taxa than the number recorded in this study (Masese et al. 2009b). River Nzoia water quality has also been found to be significantly impacted by agriculture and land use practices in the upper catchment of the river system (Osano et al. 2003; Masese et al. 2009a). These pollutant loads might have compounded or exacerbated the effects of the mill’s wastewater discharges, resulting in the overall low taxon numbers at the two sites. This can be confirmed by the fact that the Ephemeroptera taxa recorded at the upstream site (e.g. *Baetis* sp., *Philopotamus* sp., and *Caenis* sp.), are among the most tolerant groups to eutrophication (Thorne and Williams 1997), as is the case also for most Cyanophytes (APHA 1998). However, the marked shift in composition from pollution sensitive taxa at the upstream site to pollution tolerant taxa at the downstream site is indicative of the additional effects of the pulp mill effluents on macroinvertebrate and phytoplankton assemblages. Findings in this study are in agreement with results from studies elsewhere on the effects of pulp mill effluent on macroinvertebrate taxa such as *Chironomus* and *Microcystis* was increased while intolerant groups were either eliminated or their abundance was considerably depressed downstream of the mill outfall, may be a clear indication of the impact of PanPaper Mills’ effluent on River Nzoia.

**Conclusion**

From this study it can be concluded that: (1) PanPaper Mills produces effluents that change both physicochemical parameters of the receiving water and contribute to nutrient loading, especially phosphorus and nitrate; (2) the mill effluent affects the taxon richness and abundance of both phytoplankton and macroinvertebrates; and (3) the deteriorating water quality and eutrophication eliminates some taxa of both phytoplankton and macroinvertebrates, whereas others such as *Microcystis* sp. and *Chironomus* sp. appear to thrive due to their tolerance to changing water quality. It is therefore recommended that water quality monitoring that involves effluents of this nature be done using a combination of chemical analysis and biological indicators such as phytoplankton and macroinvertebrates.

**Acknowledgements**

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**References**


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1995–2009: What Have We Learned About Effluent Biotreatment in Relation to Environmental Protection?

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FPInnovations, 570 boul. Saint-Jean, Pointe-Claire, Quebec, Canada H9R 3J9

Since 1995, most mills in Canada have biotreatment in order to meet effluent regulatory limits for toxicity, biochemical oxygen demand, and total suspended solids. With occasional exceptions, the limits have been met. However, questions remain about effluent biotreatment regarding environmental protection, such as the reproductive capacity of fish. To address these concerns, a series of before-after studies were undertaken during the past decade. These included i) comparisons of effluents before and after biotreatment by means of fish (vitellogenin activity and egg production) and Ceriodaphnia (young production) tests done in the laboratory and ii) comparisons of fish communities in a river before and after the installation of effluent biotreatment at two mill sites. In all laboratory tests and with respect to all endpoints examined in these tests, the effects of the effluents after biotreatment were less or nonexistent when compared with the effects of the effluents before biotreatment. The assessment of the fish communities based on various metrics (e.g., percent piscivores, percent fish with anomalies) indicated improved conditions after the installation of biotreatment. Taken overall, the results indicated that biotreatment has improved effluent quality and this has resulted in clear improvements for the receiving environment.

Key words: pulp mill effluents, biotreatment, laboratory tests, fish communities, fish reproduction, endocrine disruption

Introduction

In Canada, the Pulp and Paper Effluent Regulations were revised in 1992 (Fisheries Act 1992). The revised regulations set stricter biochemical oxygen demand (BOD) and total suspended solids (TSS) limits. As well, there was a requirement that the effluents, at 100% concentration, cause no more than 50% mortality in 96-h tests with rainbow trout (Oncorhynchus mykiss). The revised regulations necessitated the installation of effluent biotreatment at mills that had no such facilities, or upgrades at some of the mills with existing biotreatment facilities. Through effluent biotreatment, the industry was able to achieve regulatory compliance with only occasional exceptions (Kovacs et al. 2002a).

While regulatory compliance through biotreatment is a noteworthy achievement, the ultimate goal is to protect the environment and ensure the survivability, growth, and reproduction of aquatic organisms living in waters receiving effluents from pulp and paper mills. One of the key concerns regarding mill effluents is the potential to alter fish reproduction, possibly through endocrine disruption, and thereby influence fish populations/communities (Lowell et al. 2005). What is the role of effluent biotreatment when it comes to effluent-related effects on fish reproduction? The available information from research studies that could answer this question is scarce and contradictory. There have been reports that effluent biotreatment reduces effects, but there have also been reports of no benefit and even worsening of effluent quality (Hewitt et al. 2008). Aside from research studies, another source of information about mill effluents may be the Environmental Effects Monitoring (EEM) studies. The revised Canadian regulations require the completion of EEM studies at each mill site every three years. The goal is to look for effects of mill effluents on wild organisms and use the information to evaluate the adequacy of the regulations on a site-specific basis. The EEM includes an assessment of wild fish upstream and downstream from mill discharges. In addition, mill effluent must be tested in the laboratory for sublethal toxicity to algae, invertebrates, and fish. One of the invertebrate tests examines the effects of effluents on Ceriodaphnia reproduction. To date, four EEM cycles have been completed and this covers the period of biotreatment installation and upgrades in Canada. For fish, the national dataset for each cycle was subjected to meta analysis (Lowell et al. 2005). The meta analysis showed a national pattern for effluent-exposed fish: larger livers and condition factors but smaller gonads. This is interpreted as effluent-related metabolic disruption with the potential to jeopardize fish reproduction. While the national pattern observed for several cycles has diminished with time (Tessier et al. 2009), no analysis of the data has been made to assess specific situations at individual mill sites before and after the installation or upgrade of biotreatment systems. In terms of sublethal toxicity tests, there has been a dramatic improvement in effluent quality, including reduced effects on Ceriodaphnia reproduction, between the first and second EEM cycles (Scroggins et al. 2002) when most of the changes in effluent treatment occurred. However, there was no comparison of effluent toxicity before and after biotreatment that would have allowed a direct measure of biotreatment performance.

The assessment of the role of effluent biotreatment requires studies specifically designed for this purpose. These need to include laboratory tests with direct

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comparisons of reproductive capacity of aquatic organisms (currently of greatest concern) exposed to mill effluents before and after biotreatment, as well as the appraisal of wild fish downstream from mill discharges before and after the installation of biotreatment facilities. Such before-after studies have been done at FPInnovations (formerly Paprican) as part of a larger effort aimed at monitoring effluent quality in relation to changes in mill operating conditions over time. The aim here is to collate the information related to effluent biotreatment, some of which has already been published (Kovacs et al. 2002b; Martel et al. 2008), to ascertain the role of biotreatment beyond regulatory compliance, particularly regarding its role in the protection of the aquatic environment.

**Methods**

**Laboratory Tests**

**Test species:** The three species used for testing in the laboratory included the fathead minnow (Pimephales promelas), rainbow trout, and Ceriodaphnia dubia. The fathead minnow and the rainbow trout were maintained in well water (pH approximately 8.3, hardness approximately 250 mg/L as CaCO$_3$ and alkalinity approximately 160 mg/L as CaCO$_3$). The *Ceriodaphnia* were maintained in a mixture of well water (70%) and distilled water (30%).

**Adult fathead minnow reproduction test:** The tests with 10- to 16-month-old fathead minnows were done under flow-through conditions at 25 ± 1°C and followed two published protocols (Ankley et al. 2001; Kovacs et al. 2007). The main difference in the two protocols was test duration. In one, the fish were kept under preexposure conditions for three weeks followed by a three-week exposure to effluents. The detailed conditions for these tests have been published by Martel et al. (2008). In the tests of shorter duration, both the preexposure and effluent-exposure periods were reduced to five days (Kovacs et al. 2007). Otherwise, the test protocols were similar to the longer-term tests. There were four replicates of each control and effluent-exposed groups. During the experiments, the fish were monitored for egg production.

**Rainbow trout vitellogenin tests:** Eight immature rainbow trout (weight 0.5 to 1.4 g) were exposed to 15- to 17-L volumes of effluents for seven days under static renewal conditions. The full test volume was replaced daily. The loading densities (0.07 to 0.18 g/L), aeration rates (6.5 cm$^3$ per L per min), dissolved oxygen (>70% saturation), and pH (6.5 to 8.5) were all in keeping with Environment Canada (2000) protocol for acute lethality toxicity tests with trout. Test temperature was 13 ± 1°C.

At the end of each test, the fish were sacrificed and measured for length and weight. The carcasses were homogenized individually in phosgel buffer (0.04 M Na$_2$HPO$_4$, 0.009 M NaH$_2$PO$_4$, 0.1% gelatin, and 0.0002 M Thimersol; pH 7.6) at 4°C and centrifuged at 3100×G for 10 minutes, also at 4°C. The resulting supernatants were stored at -80°C for vitellogenin analysis.

Analysis of vitellogenin (VTG) was conducted using the rainbow trout EIA (enzyme immunoassay) kit from Biosense Laboratories (Bergen, Norway). This enzyme-linked immunosorbent assay (ELISA) uses the specific binding of antibodies with vitellogenin to quantify the amount of the vitellogenin in individual fish samples (blood and whole body homogenates). Whole-body homogenates were used in this study as it was not possible to obtain sufficient blood samples for VTG analysis from the fish weighing 0.5 to 1.4 g.

**Ceriodaphnia dubia reproduction tests:** The tests with *Ceriodaphnia dubia* followed Environment Canada (1992) static renewal protocols. The tests were done in 30-mL plastic containers (10 replicates) with full test volume replacements every day. The test endpoint is survival and young production. The tests were terminated when at least 60% of the controls had three broods of young (neonates), which occurred within seven days.

**Effluents:** The details of the mills selected for effluent sampling are shown in Table 1. At each mill site, the effluents were sampled simultaneously before and after biotreatment by mill staff. In order to reduce the workload for mill staff, the collection of the effluents before and after biotreatment was not done in a staggered manner that took into account actual treatment times. As such, an assumption was made that the simultaneous sampling accurately reflected biotreatment performance. This assumption was strengthened by the knowledge that, at the time of sampling, the mills were operating under steady-state conditions.

Effluents from five mills were sampled for the fathead minnow egg production tests, and effluents were sampled from 12 mills (four of the 12 mills were the same as for the fathead minnow tests) for the rainbow trout VTG tests and the *C. dubia* reproduction tests (see Table 1). The mills represented the major types of pulp manufacturing processes and effluent biotreatment facilities in Canada. The fathead minnow tests were performed with effluents from three bleached kraft (BK) mills and two thermomechanical pulp (TMP) mills. The BK mill effluents were treated in aerated stabilization basins and the TMP mill effluents were treated in activated sludge plants, including a sequential batch reactor. The *C. dubia* and the rainbow trout VTG tests were performed with effluents from five TMP mills, one bleached chemithermomechanical pulp (BCTMP) mill, four kraft mills, and two multiprocess (MP) mills that employed more than one pulping process (e.g., kraft and TMP). The effluents at the TMP/BCTMP mills were treated in activated sludge plants, including a sequential batch reactor. The kraft mill effluents were treated in...
aerated stabilization basins and oxygen activated sludge (OAS) plants, while the effluents from the multiprocess mills were treated in OAS plants. Each effluent before biotreatment was tested at three or four concentrations and each effluent after biotreatment was tested at four to five concentrations. For the effluents before biotreatment, the concentrations for the fathead minnow, *Ceriodaphnia*, and trout VTG tests ranged from 1 to 65%, 0.1 to 28%, and 1 to 20%, respectively. For the same three tests, the biotreated effluent concentrations ranged from 2 to 100%, 1 to 100%, and 2 to 100%, respectively.

**Statistical analysis:** All the statistical analyses and toxicity threshold estimations followed guidelines provided by Environment Canada (2005) using CETIS v1.7.0revW software (Tidepool Scientific Software) or Statgraphics Centurion XV software (StatPoint Inc.). For the fathead minnow and *Ceriodaphnia* reproduction tests, the inhibition concentration causing a 25% decrease (IC25) in the number of eggs and young produced, respectively, was estimated by monotonic smoothing and linear interpolation, with the 95% confidence intervals calculated by a bootstrap method. The trout VTG data were tested for normality and homogeneity. Effluent-related effects were determined by analysis of variance (ANOVA) at \( p < 0.05 \). In cases when the ANOVA indicated a significant effluent-related effect, the Dunnett's test was used to identify the specific effluent concentrations (including the lowest observable effect concentration or LOEC) that were significantly different from the control. The threshold observed effect concentration (TOEC) was calculated as the geometric mean of the no observed effect concentration (NOEC) and the LOEC.

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**TABLE 1. Description of the mills selected for study and biological tests conducted with effluents from these mills**

<table>
<thead>
<tr>
<th>Mill</th>
<th>Process</th>
<th>Wood furnish</th>
<th>Bleaching or brightening</th>
<th>Treatment system</th>
<th>Water usage (m³/d)</th>
<th>Laboratory tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BK</td>
<td>Hardwood</td>
<td>DEoD(P)</td>
<td>ASB</td>
<td>43</td>
<td>FHM reproduction, RT VTG, Cd reproduction</td>
</tr>
<tr>
<td>2</td>
<td>BK</td>
<td>Softwood</td>
<td>DEoDEd</td>
<td>ASB</td>
<td>59</td>
<td>FHM reproduction, RT VTG, Cd reproduction</td>
</tr>
<tr>
<td>3</td>
<td>BK</td>
<td>Softwood (SW) and Hardwood (HW) (two separate lines)</td>
<td>SW: O-DEoDnD HW: O-ZDEoDnD</td>
<td>ASB</td>
<td>115</td>
<td>FHM reproduction</td>
</tr>
<tr>
<td>4</td>
<td>TMP</td>
<td>Softwood, 97% and Hardwood, 3% and Denked Fibre</td>
<td>Hydrosulphite</td>
<td>SBR</td>
<td>32</td>
<td>FHM reproduction, RT VTG, Cd reproduction</td>
</tr>
<tr>
<td>5</td>
<td>TMP</td>
<td>Softwood</td>
<td>Hydrosulphite</td>
<td>AS</td>
<td>21</td>
<td>FHM reproduction, RT VTG, Cd reproduction</td>
</tr>
<tr>
<td>6</td>
<td>TMP</td>
<td>Softwood 96% and Hardwood 4%</td>
<td>Hydrosulphite</td>
<td>AS</td>
<td>32</td>
<td>RT VTG, Cd reproduction</td>
</tr>
<tr>
<td>7</td>
<td>TMP</td>
<td>Softwood</td>
<td>Hydrosulphite</td>
<td>AS</td>
<td>47</td>
<td>RT VTG, Cd reproduction</td>
</tr>
<tr>
<td>8</td>
<td>TMP</td>
<td>Softwood</td>
<td>Hydrogen peroxide, hydrosulphite</td>
<td>AS</td>
<td>32</td>
<td>RT VTG, Cd reproduction</td>
</tr>
<tr>
<td>9</td>
<td>BCTMP</td>
<td>Hardwood</td>
<td>Hydrogen peroxide, sodium silicate</td>
<td>AS</td>
<td>22</td>
<td>RT VTG, Cd reproduction</td>
</tr>
<tr>
<td>10</td>
<td>BK</td>
<td>Softwood</td>
<td>O-D(EP)DEpD</td>
<td>ASB</td>
<td>39</td>
<td>RT VTG, Cd reproduction</td>
</tr>
<tr>
<td>11</td>
<td>BK</td>
<td>Softwood, 90% and Hardwood, 10%</td>
<td>DNED, DED</td>
<td>OAS</td>
<td>88</td>
<td>RT VTG, Cd reproduction</td>
</tr>
<tr>
<td>12</td>
<td>MP</td>
<td>Softwood, 80% and Hardwood, 20% (one kraft line and one mechanical pulp line)</td>
<td>DEoDEd</td>
<td>OAS</td>
<td>73</td>
<td>RT VTG, Cd reproduction</td>
</tr>
<tr>
<td>13</td>
<td>MP</td>
<td>Softwood, 37% and Hardwood, 63%</td>
<td>Hydrogen peroxide</td>
<td>AS</td>
<td>68</td>
<td>RT VTG, Cd reproduction</td>
</tr>
</tbody>
</table>

<sup>a</sup> BK = bleached kraft pulping; TMP = thermomechanical pulping; BCTMP = bleached chemi-TMP; MP = multiprocess – more than one pulping process.

<sup>b</sup> Bleaching terminology: D = chlorine dioxide; E = caustic extraction; o = oxygen; O = oxygen delignification; p or P = peroxide; Z = ozone; n or N = neutralization.

<sup>c</sup> ASB = aerated stabilization basin; SBR = sequential batch reactor; AS = activated sludge; OAS = oxygen activated sludge.

<sup>d</sup>FHM = fathead minnow; RT VTG = rainbow trout vitellogenin; Cd = *Ceriodaphnia dubia.*
Field Studies

Study area and mill descriptions: The field studies were done in the portion of the St. François River receiving discharges from three pulp and paper mills (Fig. 1), and details of the study area have been published previously (Kovacs et al. 2002b). The upstream and downstream stretches of the river at each mill site are separated by a dam. This resulted in some habitat differences related to maximum width/depth, flow, and dominant substrate (clay, sand, and gravel upstream and alluvium, gravel, sand, and bedrock downstream). The mill at East Angus produces about 230 t/d of unbleached kraft pulp from softwood chips. The mill effluent is treated (along with municipal effluent) in a three-cell aerated stabilization basin installed in 1995. The mill at Bromptonville produces about 700 t/d of TMP newsprint from softwood chips. The mill also operates a deinking plant and incorporates about 25 to 30% recycled furnish into its pulp stock. The pulp is brightened with sodium hydrosulphite. The effluent is treated in a sequential batch reactor (since 1995) with a retention time of approximately 5 h. The mill at Windsor produces specialty papers from hardwood bleached kraft pulp. The bleaching sequence is DEoD(P) (see Table 1 for bleaching terminology definitions) and the daily production is roughly 1,600 t. The effluent is treated (since 1987) in an aerated stabilization basin with a 5-d retention time.

Fish community assessment: Fish were sampled during August 1998 by electric shocker along 500 m of shoreline on both sides of the river upstream and downstream from the mill effluent discharges (Fig. 1). The sampling sites corresponded to sampling sites of a 1991 study done during August to mid-September (Richard 1996) prior to the existence of biotreatment facilities at the East Angus and Bromptonville mills. This allowed a direct comparison of fish communities before and after the 1995 installation of the biotreatment systems at these two sites. Since the third mill on the river at Windsor already had biotreatment system since 1987, the fish communities at this site offered another opportunity for comparison at a site where the situation was relatively stable in terms of effluent quality between 1991 and 1998. Each fish was identified by species and their lengths and weights were recorded. A maximum of 20 fish from each site were examined for external anomalies such as fin erosion, lesions, tumours, and the presence of parasites. The fish communities were characterized by the following metrics: percent omnivores, percent insectivores, percent piscivores, percent fish with anomalies, number of Catostomidae, and number of pollution intolerant species.

Results

Laboratory Tests

Adult fathead minnow reproduction test. The egg production data for the tests with the effluents from the five mills are summarized in Table 2. For the three kraft mills, the effluents before biotreatment caused a 25% reduction in egg production (IC25) at concentrations of approximately 3 to 32%. The IC25s for the biotreated kraft mill effluents were 29, 48, and >40%. This represents a 1.5-fold (Mill 3) to more than 11-fold (Mill 1) improvement in effluent quality. For the two TMP mills, the IC25s of the effluents before biotreatment were <1 to 2%. The IC25s for the biotreated TMP effluents were 24 and 27%, representing a 48- and 68-fold reduction in effluent-related effects, respectively. The average IC25 of the effluents before biotreatment was 8%, and after biotreatment it was 34%, representing a 4-fold overall decrease in effects.

Rainbow trout vitellogenin tests: Virtually all of the effluents before biotreatment caused a statistically significant induction of VTG activity in immature rainbow trout (see Table 3). The TOEC for the 12 effluents before biotreatment ranged from 1 to 14%. There was little difference in the TOEC for these effluents originating from mills using different processes and biotreatment systems. After biotreatment, the TOEC of the effluents increased in the range of 28 to >100%, representing 7- to 100-fold reductions in the VTG response (a TOEC of >100% was assumed to be 100%). The average TOEC for the VTG effect for effluents before biotreatment was 4.6%, while after biotreatment it was 83%. This represents an 18-fold reduction in effects based on averages.

Ceriodaphnia dubia reproduction tests. The results of the Ceriodaphnia tests are shown in Table 4. The IC25s
### TABLE 2. The effect of five mill effluents before and after biotreatment on the egg production of fathead minnow

<table>
<thead>
<tr>
<th>Mill</th>
<th>Process&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Biotreatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>IC25 (%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before biotreatment</td>
</tr>
<tr>
<td>1</td>
<td>BK</td>
<td>ASB</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>BK</td>
<td>ASB</td>
<td>3.4</td>
</tr>
<tr>
<td>3</td>
<td>BK</td>
<td>ASB</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>TMP</td>
<td>SBR</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>TMP</td>
<td>AS</td>
<td>0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> BK = bleached kraft pulping; TMP = thermomechanical pulping.  
<sup>b</sup> ASB = aerated stabilization basin; SBR = sequential batch reactor; AS = activated sludge.  
<sup>c</sup> IC25 = effluent concentration required to cause a 25% reduction in egg production.

### TABLE 3. The effect of 12 mill effluents before and after biotreatment on vitellogenin levels in whole-body homogenates of rainbow trout

<table>
<thead>
<tr>
<th>Mill</th>
<th>Process&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Biotreatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>TOEC (%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before biotreatment</td>
</tr>
<tr>
<td>1</td>
<td>BK</td>
<td>ASB</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>BK</td>
<td>ASB</td>
<td>&lt;2</td>
</tr>
<tr>
<td>4</td>
<td>TMP</td>
<td>SBR</td>
<td>4.5</td>
</tr>
<tr>
<td>5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>TMP</td>
<td>AS</td>
<td>&lt;2</td>
</tr>
<tr>
<td>6</td>
<td>TMP</td>
<td>AS</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>TMP</td>
<td>AS</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>TMP</td>
<td>AS</td>
<td>&gt;2</td>
</tr>
<tr>
<td>9</td>
<td>BCTMP</td>
<td>AS</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>BK</td>
<td>ASB</td>
<td>4.5</td>
</tr>
<tr>
<td>11</td>
<td>BK</td>
<td>OAS</td>
<td>4.5</td>
</tr>
<tr>
<td>12</td>
<td>MP</td>
<td>OAS</td>
<td>1.4</td>
</tr>
<tr>
<td>13</td>
<td>MP</td>
<td>AS</td>
<td>4.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> BK = bleached kraft pulping; TMP = thermomechanical pulping; BCTMP = bleached chemical TMP; MP = multiprocess.  
<sup>b</sup> ASB = aerated stabilization basin; SBR = sequential batch reactor; AS = activated sludge; OAS = oxygenated activated sludge.  
<sup>c</sup> TOEC = threshold observed effect concentration (geometric mean of NOEC and LOEC).  
<sup>d</sup> Effluents from this mill were only tested at one concentration (2% before biotreatment and 100% after biotreatment).

### TABLE 4. The effect of 12 mill effluents before and after biotreatment on the reproduction of Ceriodaphnia dubia<sup>a</sup>

<table>
<thead>
<tr>
<th>Mill</th>
<th>Process</th>
<th>Biotreatment</th>
<th>IC25 (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before biotreatment</td>
</tr>
<tr>
<td>1</td>
<td>BK</td>
<td>ASB</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>BK</td>
<td>ASB</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td>TMP</td>
<td>SBR</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>TMP</td>
<td>AS</td>
<td>0.01</td>
</tr>
<tr>
<td>6</td>
<td>TMP</td>
<td>AS</td>
<td>0.07</td>
</tr>
<tr>
<td>7</td>
<td>TMP</td>
<td>AS</td>
<td>0.13</td>
</tr>
<tr>
<td>8</td>
<td>TMP</td>
<td>AS</td>
<td>0.01</td>
</tr>
<tr>
<td>9</td>
<td>BCTMP</td>
<td>AS</td>
<td>0.4</td>
</tr>
<tr>
<td>10</td>
<td>BK</td>
<td>ASB</td>
<td>3.0</td>
</tr>
<tr>
<td>11</td>
<td>BK</td>
<td>OAS</td>
<td>4.9</td>
</tr>
<tr>
<td>12</td>
<td>MP</td>
<td>OAS</td>
<td>1.3</td>
</tr>
<tr>
<td>13</td>
<td>MP</td>
<td>AS</td>
<td>2.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> For abbreviations see Table 3.  
<sup>b</sup> IC25 = effluent concentration causing a 25% reduction in young production.
for the effluents before biotreatment ranged from 0.01 to 20%. The greatest effects were caused by the effluents from the TMP/BCTMP mills, with IC25s ranging from 0.01 to 0.4%. The IC25s for the kraft mills ranged from 2.7 to 20%. The IC25s for the biotreated effluents ranged from 9.9 to >100%, representing 2.3- to 4,348-fold reductions of effluent-related effects (an IC25 of >100% was assumed to be 100%). The greatest reductions were seen for the TMP effluents because of the very low IC25s of the effluents before biotreatment. After biotreatment, there was little difference in effluent quality from mills representing different pulping processes and biotreatment systems. The effluents before biotreatment had an average IC25 of 2.7%, and the effluents after biotreatment had an average IC25 of 48%, representing an 18-fold reduction in effects based on averages.

### Field Studies

The fish community characteristics upstream and downstream from three mills in 1998 (Kovacs et al. 2002b) are shown in Table 5. At each site, there were 11 to 16 species and 185 to 758 individuals. At all downstream sites the catch per unit effort and the total number of individuals captured were lower. It was easier to capture fish at the upstream sites which were above dams due to the slower flows. As well, there were fewer species downstream from the East Angus and Bromptonville mills and an increase downstream from the Windsor mill. Again, this may have been the consequence of differences in habitat above and below the dams.

The composition of the fish communities was assessed on the basis of metrics described by Richard

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Trophic level</th>
<th>Pollution tolerance</th>
<th>Sampling locations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>East Angus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Up</td>
</tr>
<tr>
<td>Catostomidae</td>
<td>Catostomus catostomus</td>
<td>INS</td>
<td>INR</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Catostomus commersoni</td>
<td>OMN</td>
<td>TOL</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Moxostoma anisurum</td>
<td>INS</td>
<td>INR</td>
<td>4</td>
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<td>Moxostoma macrolepidotum</td>
<td>INS</td>
<td>INR</td>
<td>7</td>
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<td></td>
<td>Moxostoma valenciennesi</td>
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<td>0</td>
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<td>Centarchidae</td>
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<td>PIS</td>
<td>INR</td>
<td>23</td>
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<td>Lepomis gibbosus</td>
<td>INR</td>
<td>INR</td>
<td>26</td>
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<td>Micropterus dolomiei</td>
<td>PIS</td>
<td>INR</td>
<td>15</td>
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<td>Cyprinus carpio</td>
<td>OMN</td>
<td>TOL</td>
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<tr>
<td></td>
<td>Lucilus cornutus</td>
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<td>INR</td>
<td>3</td>
</tr>
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<td></td>
<td>Notemigonus crysoleucus</td>
<td>OMN</td>
<td>TOL</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Notropis atberionides</td>
<td>INS</td>
<td>INR</td>
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<td>Pimephales notatus</td>
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<td>INR</td>
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<td>Gasterosteida</td>
<td>Culaca inconstans</td>
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<td>INR</td>
<td>0</td>
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<td>Hiodon tergisus</td>
<td>INS</td>
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<td>0</td>
</tr>
<tr>
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<td>Etheostoma olmsti</td>
<td>INS</td>
<td>INR</td>
<td>9</td>
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<tr>
<td></td>
<td>Perca flavescens</td>
<td>PIS</td>
<td>INR</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>Percina caprodes</td>
<td>INS</td>
<td>INR</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Percina copelandi</td>
<td>INS</td>
<td>INT</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Stozestesid vitreum</td>
<td>PIS</td>
<td>INR</td>
<td>1</td>
</tr>
<tr>
<td>Petromyzontida</td>
<td>Ichthyomyzon unicuspis</td>
<td>PAR</td>
<td>INR</td>
<td>1</td>
</tr>
</tbody>
</table>

### Electrofishing data

- Total number of fish: 758, 243, 683, 227, 236, 185
- Total number of species: 16, 12, 15, 11, 14
- Catch per unit effort: 13.7, 7.1, 14.5, 5.2, 4.5, 4.2

---

*Data from Kovacs et al. 2002b.*

*PIS = piscivore; INS = insectivore; OMN = omnivore.*

*TOL = pollution tolerant; INR = intermediate; INT = pollution intolerant.*

*Up = upstream; Down = downstream.*
Benefits of Effluent Biotreatment

(1996), which originated from metrics developed by Karr (1981) for estimating the Index of Biotic Integrity. The metrics help define the status of the fish communities. Specifically, the fish community is considered to be improved if there is i) an increase in the percent piscivores, percent insectivores, number of Catostomidae, and number of pollution intolerant species, and ii) a decrease in the percent omnivores and percent of fish with anomalies. A comparison of the metrics of the fish communities at the six sampling locations between 1991 (before the existence of biotreatment facilities at the East Angus and Bromptonville mills) and 1998 is presented in Table 6. The following differences at the East Angus and Bromptonville mill sites were evident between 1991 and 1998:

- At downstream sites, a roughly i) six-fold decrease in the percent omnivores and percent of fish with anomalies, ii) three-fold increase in the percent insectivores and piscivores at East Angus, and iii) two- and seven-fold increases in the percent piscivores at East Angus and Bromptonville, respectively.
- At upstream sites from the two mills, i) less than two-fold changes in the percent omnivores, percent insectivores, percent piscivores, and percent fish with anomalies at East Angus, and roughly ii) a three-fold decrease in the percent omnivores, two-fold decrease in the percent insectivores, and six-fold increase in the percent piscivores at Bromptonville as well as an increase in the percent of fish with anomalies from 0 to 3.3%.
- Little change in the number of pollution intolerant species and Catostomidae at all the sites.

At Windsor (see Table 6), where effluent biotreatment was in existence since 1987, the following highlights the comparisons between 1991 and 1998:

- At the downstream site, i) a three-fold decrease in percent omnivores, ii) less than a 1.5-fold increase in percent insectivores and piscivores, and iii) less than a two-fold decrease in percent of fish with anomalies.
- At the upstream site, i) a five-fold decrease in percent omnivores, ii) a six-fold decrease in percent insectivores, and iii) a six-fold increase in percent piscivores.

TABLE 6. Comparison of fish community characteristics in the St. François River between 1991 and 1998 upstream and downstream from three pulp and paper mills *

<table>
<thead>
<tr>
<th>Fish community indicators</th>
<th>Sampling locations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>East Angus</td>
</tr>
<tr>
<td>% omnivores</td>
<td>11/6 72/11</td>
</tr>
<tr>
<td>% insectivores</td>
<td>51/73 25/77</td>
</tr>
<tr>
<td>% piscivores</td>
<td>39/21 4/9</td>
</tr>
<tr>
<td>% anomalies</td>
<td>1.8/0.3 14/2.6</td>
</tr>
<tr>
<td>No. of intolerant species</td>
<td>1/1 1/1</td>
</tr>
<tr>
<td>No. of Catostomidae</td>
<td>2/3 4/3</td>
</tr>
</tbody>
</table>


The overall assessment of the fish communities in 1998 revealed the following:

- At East Angus, the fish community downstream from the mill had a greater percent omnivores and percent fish with anomalies, and a lower percent piscivores. The percent insectivores, number of intolerant species, and Catostomidae were about the same upstream and downstream from the mill.
- At Bromptonville, the fish community downstream from the mill had a lower percent insectivores and number of Catostomidae, but a greater percent piscivores and number of intolerant species. The percent of the fish with anomalies and percent omnivores were about the same.
- At Windsor, the fish community downstream from the mill had increased i) percent insectivores, ii) number of intolerant species, iii) number of Catostomidae, and iv) percent fish with anomalies, and decreased percent piscivores. The percent omnivores was about the same.

**Discussion**

Effluent biotreatment has been the cornerstone of the strategy employed by the Canadian pulp and paper industry for compliance with environmental regulations. While the strategy has been very successful regarding regulatory compliance, there has been relatively little information available as to the specific role of biotreatment concerning overall effluent quality relating to the protection of the aquatic environment. The collation of the information in this paper from the before-after biotreatment studies conducted over a period of more than 10 years indicates that effluent biotreatment had demonstrable benefits, beyond regulatory compliance, both in terms of tests done in the laboratory to assess effluent quality and in terms of the assessment of wild fish communities in a river.
Laboratory Tests

The laboratory tests were done with effluents from 13 mills representing the major types of pulp/paper manufacturing processes and types of biotreatment systems in Canada. The three laboratory tests were selected for the purpose of best assessing the effects of effluents on the reproductive capacity of aquatic organisms, including endocrine disruption. Specifically, egg production by the fathead minnow has been found to be the most sensitive indicator of effluent-related reproductive effects (Parrott 2005), the VTG induction in immature fish such as rainbow trout is considered to be a standard test for endocrine disruption (Hiramatsu et al. 2006; Jones et al. 2000), and young production by Ceriodaphnia is a good indicator of reproduction by invertebrates (Environment Canada 1992). These laboratory tests provided strong evidence regarding the benefits of effluent biotreatment on effluent quality. Specifically, biotreatment reduced/eliminated mill effluent-related effects in terms of i) egg production by the fathead minnow, ii) induction of VTG in rainbow trout, and iii) young production by Ceriodaphnia. These improvements were observed for all 13 effluents representing different manufacturing processes and types of biotreatment and for all the endpoints. In no case was there either no improvement in effluent quality after biotreatment or a worsening of effects.

While the benefit of biotreatment on effluent quality was noted in all cases, there were differences in the degree of the benefits. The degree of the benefits could depend on many factors, such as the manufacturing process (both pulping and bleaching/brightening), the wood furnish, the type of effluent biotreatment (e.g., activated sludge, aerated stabilization basin), the quality of the effluent before biotreatment, and water usage. However, this series of investigations showed that the degree of benefit is not exclusively linked to any one of these factors. In fact, the manufacturing process (including wood furnish) and the type of biotreatment had no consistent role regarding the quality of the biotreated effluents (see Tables 2 to 4). For example, activated sludge (one of the most commonly used biotreatment systems in Canada) and oxygen activated sludge treatment completely eliminated (i.e., IC25 >100%) the effects of effluents from a TMP and kraft mill (representing the major types of pulping processes in Canada), respectively, on the reproduction of Ceriodaphnia. On the other hand, at another TMP mill, the IC25 of the biotreated (also activated sludge) effluent in a Ceriodaphnia test was only 25%. For kraft mills (using various forms of elemental chlorine free bleaching sequences), the effluent treated in aerated stabilization basins (common means of treatment for kraft mill effluents) had IC25s of 9.9 and 46% in tests with the Ceriodaphnia. Prior to biotreatment at these two kraft mills, the IC25 of the effluents was 2.7 and 20%, respectively, suggesting that in these cases, the quality of the effluent before biotreatment may have influenced the quality of the effluent after biotreatment. However, in the fish reproduction tests, there were examples where the effluent quality before biotreatment was the same, yet there was substantial difference in effluent quality after biotreatment (see IC25s for mills 1 and 2 in Table 2).

Since none of the mill conditions (e.g., pulping process, wood furnish) considered in this work could fully explain the differences in the degree of benefits associated with biotreatment, it is clear that the final effluent quality from a mill is the consequence of a complex set of factors. The most favourable mill operating conditions achieving the best quality of effluent remains to be identified in future studies. In the meantime, specific mill operating conditions that i) minimize the prevention of loss of organic material to sewer (e.g., spill control) and ii) maximize the efficiency of the biotreatment systems are the best leads for minimizing risks associated with mill effluents (Kovacs et al. 2009).

In general, the TMP mill effluents before biotreatment were more toxic to Ceriodaphnia and the fathead minnow than kraft mill effluents. There are two explanations for this. One, the TMP mills use less water and this results in a greater concentration of causative agents in the effluents before biotreatment. Two, the TMP mills typically utilize softwoods such as balsam fir for making pulp. Balsam fir contains juvabiones which end up in the effluents and are extremely toxic to Ceriodaphnia (O’Connor et al. 1992). The juvabiones do not survive kraft pulping conditions (Walden et al. 1986) and they are also easily biodegraded (Leach et al. 1975; Gibbons et al. 1992). The absence of an overall difference in the quality of the biotreated effluents from kraft and TMP mills further demonstrates the efficiency of biotreatment systems in reducing/eliminating some effects even when the effluent before biotreatment has substantial effects/differences.

Previous studies testing the quality of effluents before and after biotreatment in the laboratory have also largely indicated improvements attributed to biotreatment, although not in all cases. Following is a list of laboratory studies showing improvements, which were not necessarily complete elimination of effects after biotreatment of mill effluents:

- Anaerobic/activated sludge treatment of a TMP/stone groundwood mill effluent resulted in reduced effects on hormone levels (testosterone in both males and females; 17-β-estradiol unaffected by both effluents) in brown trout (Salmo trutta) and hatchability of trout eggs (Johnsen et al. 2000; Johnsen et al. 2003).
- Female mosquitofish (Gambusia affinis) exposed to effluent from a kraft/TMP mill had reduced level of masculinization after biotreatment in a system that pretreats the TMP effluent in a moving bed bioreactor, which is then combined with the kraft mill effluent for treatment in a three-pond aerated stabilization basin (Ellis et al. 2003).
- Biotreatment (mill scale: activated sludge and anaerobic/aerobic treatment; laboratory biotreatment: simulating an aerated stabilization basin and activated sludge treatment) of effluents from four mechanical
pulp/paper mills led to decreased effluent-related effects on Ceriodaphnia reproduction and fathead minnow growth irrespective of the type of biotreatment (Gibbons et al. 1992). The IC25s of the effluents for Ceriodaphnia reproduction increased from 0.01 to 0.8% for primary-treated effluents to 5 to 37% for biotreated effluents, while the IC25s for the minnow growth tests increased from 0.3 to 5.3% for primary-treated effluents to 47 to >100% for biotreated effluents.

- In a survey of effluents from 13 mills (BK and CTMP, TMP), the mixed function oxygenase (MFO) activity was much lower in rainbow trout exposed to the biotreated effluents (activated sludge and aerated stabilization basin) than to the primary-treated effluents (Martel and Kovacs 1997). All 13 primary-treated effluents caused MFO induction at 10% concentration (most even at 5% concentration and some even at 1% concentration) whereas only three of the 13 biotreated effluents caused MFO induction at 10% concentration, and the level of induction was lower than that caused by the primary-treated effluents.

- In addition to improved effluent quality on the basis of laboratory toxicity tests, biotreatment has also been shown to significantly reduce various effluent components measured by chemical analysis (LaFleur et al. 1998). In fact, the removal efficiencies by various biotreatment systems (activated sludge and aerated stabilization basin) at over 40 pulp/paper mills in the U.S.A. have been classified as moderate to high. For monoterpenes, fatty acids, resin acids, chlorinated resin acids, chlorinated phenolics, sterols, alkylcyclopentenones, acetyl- and propionylthiophenes, and acetophenone, the removal from effluents in biotreatment systems can be as high as 97 to 100%. The removal or removal efficiency was not found to be dependent on specific types of biotreatment system(s). The only compound not found to be reduced by biotreatment in a significant manner was 1,1-dichlorodimethylsulfone and, so far, this compound has not been tested for biological effects.

Other studies showed less obvious benefits of biotreatment or none at all. In one case, goldfish were exposed to effluents from a bleached sulphite (aerated stabilization basin) and bleached kraft mill (activated sludge) before and after biotreatment (Parrott et al. 2000). The hormone production by the male gonads (as well as plasma hormone concentrations) was reduced by exposure to 100% biotreated effluent from the sulphite mill. There were no such effects caused by exposure of fish to 10% primary-treated effluent. In this case, a direct comparison of primary- and biotreated effluents at 100% was not possible because at this concentration the primary-treated effluent caused mortality. In addition, after process modifications made by the mill, including changes in biotreatment, even 100% biotreated effluent caused no effects on hormone production. This demonstrated that biotreatment itself was not worsening the effluent. In the case of the kraft mill, primary-treated effluent up to 40% concentration and 100% biotreated effluent exiting the bioreactor directly caused no effects on 11-ketotestosterone production by male gonads. However, 100% effluent exiting the secondary clarifier did reduce 11-ketotestosterone production by the males. There was no explanation given and there is no obvious reason for the difference in effluent quality between the bioreactor outlet and the outlet from the secondary clarifier. In any case, no direct comparison of effluent quality before and after biotreatment was possible because the effluent after biotreatment was tested at 100% concentration and the effluent before biotreatment was tested at 40% concentration. In a study with mummichog (Fundulus heteroclitus) exposed to 1% concentration of primary- and biotreated effluents (aerated stabilization basin) from a kraft mill, the reduction of plasma testosterone in males and females was found to be greater in the fish exposed to the biotreated effluent (Dubé et al. 2002). At the time of the study, the mill used 50% chlorine dioxide substitution to bleach kraft pulp, a practice that is no longer in existence in Canada. Also, no other effluent concentrations were tested that would have allowed for an estimation of the threshold concentration for the primary and biotreated effluents. Finally, evidence of masculinization was found in guppies (Poecilia reticulate) exposed to both primary- and biotreated (activated sludge) effluents from a kraft mill, with effects seen at 5 and 25% concentration of primary-treated effluent and only 10% concentration (not 5 and 25%) of biotreated effluent (Larsson et al. 2002).

Taken overall, direct comparisons of effluents in laboratory tests have shown that in most cases the biotreatment of effluents lead to improved effluent quality. The few cases where no clear improvements have been shown are sometimes complicated by test conditions such as testing the biotreated effluents at higher concentrations than the effluents before biotreatment or nonconcentration dependent responses.

Wild Fish Studies

The study of wild fish described here did not specifically look for reproductive alterations. While such alterations may exist in fish exposed to mill effluents in the St. François River, there was evidence for improved fish community status downstream from two mills which installed biotreatment systems. When the individual metrics described here were integrated into an Index of Biotic Integrity or IBI (Karr 1981; Richard 1996), the IBI scores increased from conditions representing poor fish community status before biotreatment (1991) downstream from the East Angus and Bromptonville mills to conditions that by 1998 could be classified as good after the installation of the biotreatment systems in 1995 (Kovacs et al. 2002b). The improvements in the fish communities reflected the vastly improved condition of effluent quality based on tests assessing fathead minnow/algae growth and Ceriodaphnia reproduction (Kovacs et
al. 2002b). No such improvements were evident at the Windsor mill site with an existing biotreatment system since 1987, where the IBI scores represented a good fish community status in both 1991 and 1998. However, there was some improvement at the upstream site of the Windsor mill between 1991 and 1998 (see Table 6), suggesting that the improvements at sites downstream from the East Angus and Bromptonville mills (both upstream from Windsor) may even have contributed to an overall improvement throughout the river.

While the overall improvements between 1991 and 1998 in fish communities at the two sites with biotreatment installations in 1995 were quite evident, the examination of individual metrics indicated that some differences between the fish communities upstream and downstream from the mills still existed in 1998 (Table 6). For example, in the case of morphological anomalies in fish, the frequency upstream from the East Angus mill was 0.3%, whereas the frequency was 2.6% downstream from the mill.

Other studies have also documented improvements or partial improvements in the condition of wild fish that were related to effluent biotreatment. One example was the general improvement in the fish communities of a river in the U.S. receiving input from several mills after the mills installed biotreatment systems during the 1970s (Weinbauer et al. 1980). In studies by Munkittrick et al. (1997, 1998, 2003), where the main focus of interest was at the level of individual fish, for example, in terms of gonad size and plasma hormone levels, some improvements in these indicators in fish living downstream from mill effluents have been observed after the installation of biotreatment, but this was not always the case and the complete elimination of responses was not seen. It is possible that the endpoints at the individual fish level (e.g., plasma hormone levels) are more sensitive than the metrics used to evaluate the status of fish communities (e.g., percent piscivores, percent fish with anomalies). As well, the improvements were seen after the mills made changes in operating conditions that included process modifications (e.g., bleaching) in addition to effluent treatment. In other words, these studies were not designed to specifically study the outcome of biotreatment installations. Similarly, in the regulatory EEM studies, also evaluating fish at the individual level, in this case exclusively on the basis of morphometric characterization (e.g., gonad size), the national pattern of fish condition, that is smaller gonads and larger livers/condition factor, continues to be reported, albeit at a reduced level (Tessier et al. 2009). To better understand the context of this, in addition to the national pattern, it may be worthwhile to examine the situation at individual mills from 1995 to the present, particularly at sites with biotreatment installations during this period.

In general, as was the case for the evidence coming from laboratory tests, the study of wild fish has also indicated benefits that can be attributed to effluent biotreatment. These benefits appeared to be greater at the fish community level than at the level of individual fish.

### Summary and Conclusions

On the basis of the FPInnovations work done in the laboratory and with wild fish, effluent biotreatment resulted in i) improved effluent quality as it relates to current concerns regarding potential effluent-related effect on the reproduction of aquatic organisms and ii) improved fish community status. The improvements in effluent quality seen in laboratory tests were not dependent on the type of manufacturing process, wood furnish, water usage, or the type of effluent biotreatment. There were differences in the degree of improvements in effluent quality from one mill to the next, but the precise reason(s) for these differences is yet unknown. Specific mill operating conditions such as spill control and biotreatment efficiency may be important factors. In the case of wild fish, while improvement in the overall communities was evident, differences in some indicators in the status of upstream and downstream fish communities continued to exist even after the installation of effluent biotreatment. The FPInnovations findings are supported by the findings of other studies found in the literature, although there are also reports indicating lesser or little benefits associated with biotreatment. One reason for this discrepancy may be the study of different endpoints (e.g., at the individual fish level versus community level in the case of wild fish studies) and different study designs (e.g., testing effluents before and after biotreatment at different concentrations).

Taken overall, the results presented here and those available from the literature indicate that biotreatment has improved effluent quality and this has resulted, or has the potential to lead to, clear improvements in the receiving environment. Residual effluent-related effects continue to be reported at some sites and the elimination of these will require further studies.

### Acknowledgements

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### References


Dubé M, MacLatchy D, Culp JM, Gillis G, Parker R, Courtenay SC, Gilman CI. 2002. Utility of mobile, field-


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The Evolution of Pulp and Paper Mill Effluent Effects Knowledge and Issues – A Career-Based Retrospective

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The two recently retired authors have a combined career experience of over 65 years in carrying out research directed at assessing effluent effects on marine and freshwater receiving waters. As such, their work directed at the environmental information needs of the forest products industry has represented a continually evolving research program. This paper reflects a history of issues and progress on pulp and paper mill effluent research before their careers began, the progression of issues and research during their careers, how questions have changed and evolved, and also looks forward to the remaining questions that need to be addressed concerning effluent effects.

Key words: pulp and paper mill effluent, experimental streams, receiving water studies, laboratory bioassays, fish reproduction

Introduction

Issues related to the discharge of wastewaters from pulp and paper mills have changed significantly over time. In the U.S., for example, the decline of the Olympia oyster (Ostreola conchapila) in Puget Sound in the early 1900s was attributed to the discharge from mills (Dinnel et al. 2009), as was low dissolved oxygen and the clogging of fish nets in the Columbia River (Carter 2006). Before effluent primary or secondary treatment, pulp mill effluent entering the Willamette River in Oregon was described as “an oxygen-gulping slime-making scourge. It destroys fish life, fouls fishing gear and fishing boats. Sometimes it churns at river’s bottom forming into rafts that rise to the surface in sluggish, foul-smelling masses of filth – There was no oxygen at all. The Willamette River was dead.” (Walth 1995).

Most mills provided effluent treatment with the addition of primary settling to remove fibres and heavy solids, either prior to or beginning in the 1950s. Early studies by the National Council for Air and Stream Improvement (NCASI) indicated the benefits of aeration lagoons as secondary treatment for biochemical oxygen demand reduction, and these were installed at mills along the Willamette River in the 1960s. By 1972 the benefits of secondary treatment were being noted in the Willamette River (Gleeson 1972). With an understanding of the benefits of secondary treatment, the Clean Water Act in 1972 required secondary treatment, and most U.S. mills had met this requirement by the mid 1970s. The Act included other provisions designed to protect the health of the aquatic environment, including a “fishable/swimmable” goal targeted to provide for the protection and propagation of fish, shellfish, and wildlife, as well as recreation.

Although the record of U.S. mills provided clear examples of improved effluent quality following the implementation of secondary treatment, there were continued calls for further research to identify whether harmful effects to aquatic organisms remained. Some of these calls were from the science-based community while others were from the public or environmental groups with sincere, but not necessarily fact-based concerns. Recent protests regarding the construction of a new state of the art pulp and paper mill and advanced wastewater treatment facility in Uruguay provide an example of how (whether fact- or emotion-based) issues continue to be raised about possible harmful effects from pulp and paper mills. Science-based questions do continue to be proposed, however, as evidenced by the attendance of international participants to the series of seven Fate and Effects Conferences. These conferences, initiated in Saltsjöbaden, Sweden, in 1991, and held most recently in Fredericton, New Brunswick, Canada, in 2009, provide examples of how science and issues have evolved over time and how there appears certainty that new questions will continue to be raised.

The two authors over the course of their careers have experienced and participated (as industry-supported scientists) in this evolution, having begun their careers either just before or just after the implementation of the Clean Water Act in the U.S. Some examples of the fish-related effluent effects questions that have been raised over time are provided in Table 1. The length of this partial list suggests that as one question is answered, another will arise, given the passage of time and new ideas or tools for assessing potential effluent effects. Indeed the concept of the “Great Pulp Mill Onion” has been evoked by one researcher, suggesting that “complex effluents are like layers of an onion. As you peel back one layer of effects you reveal another” (Hodson 2008). With this perspective in place, issues of effluent effects will
undoubtedly continue to be raised over time regardless of the sophistication of research employed.

Beyond questions about direct effluent effects on fish, there have been other questions related to effluent effects on other aspects of the aquatic community, such as periphyton or macroinvertebrates. The rationale for postulating these effects is based on combinations of toxicity, added inorganic or biosolid based nutrients, and reduced underwater light transmittance because of effluent colour.

From an industry perspective there is disappointment that changes in mill process and treatment technologies have not resulted in a lessening of public and regulatory concern regarding whether effects in receiving waters are still taking place. This may be, in part, due to the dilemma that although good science has been carried out over the past several decades, there are still substantial differences in the conclusions which have been reached by investigators. In Canada, for example, data collected through the Environmental Effects Monitoring (EEM) program has provided sufficient cause to state that “Scientists know these effluents adversely affect fish populations” and that “Pulp mill effluents affect the ability of fish to reproduce and sustain their populations.” (Envirozine 2004). Conversely, Hall et al. (2009c) reported that “following 8 y of monitoring the weight of evidence suggests an absence of instream population/community effects downstream of the mill discharges” on four U.S. receiving waters. The differences in conclusions may not be reflective of whether research for these two respective programs was good or bad, but rather that different approaches were taken to identify whether and the extent to which effluent effects were present. The remaining portions of this paper recount, through the experiences of the now retired biologist authors, how effluent effects were studied and how issues evolved over the four decades since the early 1970s. Additionally the authors provide some career-based perspectives on remaining and future information needs and a view on forests as a unique renewable resource.

## Early NCASI Aquatic Biology Research

NCASI was founded by representatives of forest products companies in 1943 as a nonprofit research organization whose purpose was to provide science-based environmental technical support to the industry. Early major efforts at addressing effluent effects concerns constituted an expression that the industry recognized the need to improve effluent quality in order to preserve the abundance and diversity of receiving water communities, and that a shared funding approach through NCASI was a means to accomplish this. Some of the earliest NCASI studies were related to assessing receiving water conditions as they were influenced by pulp and paper mill effluent discharges. One of the first technical bulletins issued by NCASI related to aquatic biology research and questions about the effects of mill effluent on fish food organisms (NCASI 1947). The early studies focused on and embraced the concept of the “web of life” with research addressing both fish and the supporting food web. The earliest studies were based on acute bioassays carried out in laboratory aquaria, but these evolved by 1971 into studies carried out in large outdoor experimental stream channels.

### Experimental Stream Studies

NCASI’s experimental streams studies were undertaken out of a desire to expand the knowledge of effluent responses from observations of direct effects on individual organisms in the laboratory to assessments in a larger more natural outdoor setting where effects could be ascertained under more realistic conditions with respect to the functioning of the “web of life.” The test systems included flowing water and stream channels that were intended to mimic either coldwater faster flowing riffle/pool stream channels or warmwater meandering streams. The streams were stocked with representative fish species, and their resulting growth, survival, and production were dependent on the health of the fish based not only on their direct effluent exposure, but also on the health of

<table>
<thead>
<tr>
<th>TABLE 1. Examples of some historical or current fish related concerns for effluent discharges to receiving waters</th>
</tr>
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<tbody>
<tr>
<td>Low dissolved oxygen stress</td>
</tr>
<tr>
<td>Acute toxicity</td>
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<tr>
<td>Chronic toxicity</td>
</tr>
<tr>
<td>Avoidance responses</td>
</tr>
<tr>
<td>Blue sac disease inducer</td>
</tr>
<tr>
<td>MFO induction</td>
</tr>
<tr>
<td>Mysterious slash like lesions in fish</td>
</tr>
<tr>
<td>Reproductive impairment</td>
</tr>
<tr>
<td>Altered energetics</td>
</tr>
<tr>
<td>Flavor impairment</td>
</tr>
<tr>
<td>Developmental abnormalities</td>
</tr>
</tbody>
</table>

* GSI = gonadosomatic index; LSI = liver somatic index; CF = condition factor.
The supporting macroinvertebrate food webs as they might be influenced by effluent exposure.

The first of these studies initiated by Isaiah Gellman and Russell Blosser of NCASI, was carried out at an unbleached kraft mill in Albany, Oregon, through a cooperative agreement with Oregon State University. These studies focused on trout and salmon in the experimental streams, as well as the continued use of laboratory bioassays. The Albany experimental streams studies were supervised by Charles Warren from Oregon State University (Warren 1971), who was joined by Dennis Borton who later became NCASI’s first aquatic biology employee. The Albany studies spanned five years which included a period before and after initiation of secondary treatment of effluent at the host mill.

The focus of NCASI experimental stream research shifted in 1975 to a bleached kraft mill effluent, with Dr. Borton initiating new studies at the Southern Experimental Streams Facility in New Bern, North Carolina (Fig. 1). These studies included exposures in the streams to effluent before and after the mill completed process changes to chlorine dioxide substitution bleaching (Borton et al. 1996), and then to oxygen delignification. The studies involved year-long exposures of warmwater fish species (largemouth bass [Micropterus salmoides], bluegill sunfish [Lepomis macrochirus], golden shiner [Notemigonus crysoleucas]) and the supporting food web to biologically treated effluent at concentrations up to 13% vol/vol (NCASI 1983). No adverse effects were reported for fish based on survival, growth, production, reproduction, liver somatic index (LSI), gonadal somatic index (GSI), or condition factor (CF), or for the supporting macroinvertebrate community.

The experimental streams effort was expanded in 1980 to include a coldwater ecosystem counterpart to the Southern Experimental Streams. Tim Hall joined the NCASI staff in 1979 for the initiation of these studies at a Northern Experimental Streams Facility constructed at the site of a bleached kraft mill in Lewiston, Idaho (Fig. 2). Lewiston stream studies carried out between 1980 and 1994 focused on rainbow trout (Oncorhynchus mykiss) as a representative salmonid, with research with other salmonid species carried out in the laboratory. Studies were carried out both before (Hall et al. 1991) and after the mill underwent conversions to first increased chlorine dioxide substitution (Haley et al. 1995), and then to oxygen delignification. The Lewiston studies involved effluent exposures up to 5% vol/vol. Based on exposure periods ranging from 9 months to 3.5 years, greater levels of fish production were reported for effluent exposed fish, a likely product of similar increases noted for macroinvertebrate production. Effluent exposed rainbow trout did not, however, have significant differences in LSI or CF, and they reproduced successfully (Hall et al. 1991).

The two experimental streams studies indicated an absence of effluent effects on fish production or reproduction at concentrations considerably higher than...
the median instream effluent concentration (0.4% vol/vol) for U.S. pulp and paper mills (Beebe et al. 2004). This finding justified the conclusion of work at the Northern Streams in 1994 and the Southern Streams in 2000 with a refocus of NCASI’s aquatic biology research toward fish reproduction and endocrine disruption studies by Dr. Borton at the New Bern facility. Also at this time a research effort directed at assessing U.S. EPA (U.S. Environmental Protection Agency) whole effluent toxicity marine chronic bioassay methods, and both marine and freshwater sediment toxicity test methods, was undertaken by Mr. Hall following facility relocation to a coastal laboratory location in Anacortes, Washington.

Technical Assessment of Whole Effluent and Sediment Toxicity Test Methods

The experimental streams studies had been supported by the physical/chemical assessment of biologically important water quality parameters, the detailed characterization of the organic chemical components of the effluents tested, and newer whole effluent toxicity (WET) tests. The U.S. pulp and paper industry looked to NCASI to assist in the understanding and technical evaluation of the acute as well as newer chronic effluent WET tests being required by the U.S. EPA under the National Pollution Discharge Elimination System (NPDES) permit program. These assays included both freshwater and marine plant, invertebrate, and fish species (U.S. EPA 1995, 2002a, 2002b), NCASI’s test evaluation studies, as well as the earlier laboratory and experimental stream studies, included 7-d *Pimephales promelas* and *Ceriodaphnia dubia* freshwater growth or reproduction bioassays, and *Dendraster excentricus* and *Mytilus edulis* marine egg fertilization and embryo development bioassays. NCASI staff also became involved with the early development and interlaboratory precision testing of some of these same tests (DeGraeve et al. 1989 and Environment Canada 1992). Similar activities were also undertaken to develop practical experience with methods the U.S. EPA was developing for assessing the toxicity of both marine and freshwater sediments.

A primary theme in NCASI’s technical evaluation of bioassay methods has been to assess the adequacy of inter- and intra-laboratory precision data, and to assist in studies to fill these important information gaps when it has been found to be lacking. Substantial improvements have been made in this area as U.S. EPA and other methods have become better defined, precision tested, and more frequently used.

**Fish Reproduction Research**

Additional NCASI research to address questions about the possible direct effects of effluents on fish survival, growth, and particularly reproduction began in 1979 with the first of a series of fathead minnow life-cycle tests. The life-cycle bioassays began with the exposure of *P. promelas* eggs from laboratory cultures to various concentrations of effluent with exposure continued for an additional 5 to 6 months. Upon reaching maturity, fish were reduced to spawning “trios” with resulting egg production and other endpoints recorded (Fig. 3). The final phase of the studies were to record the hatching success and early growth and survival of the resulting offspring. Since the first of these studies was completed in 1979 (NCASI 1985), 24 fathead minnow life-cycle assays have been completed with effluents from 12 different mills, including unbleached kraft, bleached kraft, thermomechanical, deinking recycle, and cardboard container recycle mills, as well as with exposures to wood leachates, lignin, and stigmastanol (a phytosterol originating with the wood furnish). Additional multigenerational studies were also completed, including some tests before and after mill modernization. Egg production was generally the most sensitive of the reproduction metrics measured and, depending on the effluent tested, was not affected until effluent exposure reached from 8 to >100% vol/vol (NCASI 2006; Borton et al. 2009), a concentration substantially higher than the 0.4% vol/vol median concentration for U.S. pulp and paper mills (Beebe et al. 2004). Other results indicated that multigenerational exposure did not increase effects (Borton et al. 2000), mill modernization decreased effluent effects (Borton et al. 1997), effects noted were dependent on continuous exposure thus diminishing the probability of bioaccumulation (Borton et al. 2004), and that some chemical compounds originating from the wood furnish could contribute to reproduction effects, although resin acids were ruled out as contributing compounds (Borton et al. 2006). Of over 50 components measured during the life-cycle assays, polyphenols, a group of secondary metabolites naturally produced by trees, provided the highest correlation with reduced egg production (Borton et al. 2009). Also of note is that several commonly used bioindicators (e.g., GSI, LSI, CF) measured during life-cycle tests did not correlate well to egg production (Borton et al. 2003).

**Long-Term Receiving Water Studies**

The NCASI Long-Term Receiving Water Studies (LTRWS) were a logical extension of the experimental streams. Although the experimental streams offered an important step forward with respect to understanding the potential for long-term effluent exposure effects at the aquatic community/food web level, they represented rather simplified conditions compared with more complex natural streams. The concept of the LTRWS was developed by NCASI through its industry steering committee, and from this a series of scope and framework objectives was developed (Hall and Miner 1997). These objectives included the general purpose of addressing the compatibility of effluents with healthy receiving water communities, and understanding any related changes.
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following mill process modifications. The studies were designed based on their long-term timeframe (10 to 20 year) and detailed field and laboratory bioassessment to identify possible subtle effluent effects that may not have been previously identified, as well as to serve as a real world framework to assess the significance of these effects if they were identified. In addition, the length of the study and the inclusion of a watershed-scale distribution of sample sites were intended to put potential responses within the context of natural upstream/downstream variability, and variability over time. The studies, managed by Mr. Hall, were initiated in 1998/1999 at Codorus Creek (Pennsylvania), the Leaf River (Mississippi), and the Willamette/McKenzie rivers (Oregon) (Fig. 4) to represent a blend of bleached and unbleached mills, both coldwater and warmwater stream ecosystems, and a range of representative effluent concentrations (Table 2).

A key component of the study was the inclusion of a Science Advisory Panel (SAP). The six member independent panel consisted of representatives from both academia and industry with expertise in stream ecology, toxicology, and bioindicators, as well as pulp and paper mill processes. During the formative years of the LTRWS, the SAP consisted of John Rodgers (Clemson University), Wayne Landis (Western Washington University), Wayne Minshall (Idaho State University), Tibor Kovacs (FP Innovations/Paprican), Barry Firth (Weyerhaeuser), and Tom Deardorff (International Paper). Carroll (Skip) Missimer (Glatfelter) and Monique Dubé (University of Saskatchewan) joined the SAP in more recent years to fill vacancies created by the departures of Firth and Deardorff. Twice yearly meetings with NCASI staff provided direction in program development, internal quality assurance assessment, assessment of findings, and peer review of publications.

LTRWS monitoring included the assessment of fish at the whole organism and population/community level as well as similar assessments of the periphyton and macroinvertebrate communities (Fig. 5). Similar to the approach used in the experimental streams studies, instream monitoring was accompanied by the analysis of water and effluent for over 100 physical/chemical parameters, effluent chronic bioassays, streamside mesocosm studies, and fathead minnow life-cycle studies.

A series of papers was published in 2009 reporting on findings from the first eight years of the LTRWS (Borton et al. 2009; Flinders et al. 2009a, 2009b, 2009c; Hall et al. 2009a, 2009b, 2009c; Landis and Thomas 2009). Conclusions reached during this initial period of the study provide a “weight of evidence” founded on both instream and laboratory-based assessment for an absence of effluent effects for the four mill discharges studied (Table 2). That this may be more broadly applied to the larger population of mills is supported since all but one of the effluents studied was present in its receiving water at greater than the 50th percentile distribution of U.S. mills (0.4% vol/vol) (Beebe et al. 2004). One of the effluents studied (Codorus Creek mill, Pennsylvania) was actually in the >95th percentile of the distribution (33% vol/vol) and was selected as a way to address the “margin of safety” possible for other mills. Effluent chronic bioassays included in the study suggested that the “margin of safety” may have been from 2 to 3 times for Codorus Creek and from 25 to 321 times for the other three study sites.

The conclusions drawn here are substantially different from those reported in the Canadian EEM program (EnviroZine 2004; Lowell et al. 2005) where it has been stated to a high degree of certainty that paper mill effluent exposures adversely affect fish populations through metabolic disruption or reproductive impairment. The nature of the differences in conclusions may relate to the use of long-term (i.e., multiyear, multiseason) population/community-based metrics in the LTRWS (e.g., species type and relative abundance, community structure metrics such as richness and diversity, and structure and function metrics) versus less frequent (single year or periodic) individual sentinel fish

Fig. 3. Laboratory setup for conducting fathead minnow life-cycle studies (left) and spawning minnows (right).
Fig. 4. The Long-term Receiving Water Study sites at which work was initiated in 1998 to assess effluent effects on representative U.S. receiving waters. Codorus Creek (upper left), Leaf River (upper right), McKenzie River (lower left), and Willamette River (lower right).

### Table 2. Overview of LTRWS findings after the first decade: "weight of evidence"

<table>
<thead>
<tr>
<th>Mill type</th>
<th>Codorus Creek</th>
<th>Leaf River</th>
<th>McKenzie River</th>
<th>Willamette River</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleached kraft</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32.4%</td>
<td>0.8%</td>
<td>0.5%</td>
<td>0.5%</td>
<td></td>
</tr>
<tr>
<td>- edge of mixing zone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Were there significant differences downstream of the mill discharge?**

<table>
<thead>
<tr>
<th>Water quality</th>
<th>Yes</th>
<th>Yes</th>
<th>Yes</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periphyton</td>
<td>Inc</td>
<td>No</td>
<td>Inc</td>
<td>No</td>
</tr>
<tr>
<td>Macroinvertebrate</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Hilsenhof Biotic Index</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Inc</td>
</tr>
<tr>
<td>Fish</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

**What was the "margin of safety"?**

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth</th>
<th>96 h</th>
<th>2x</th>
<th>25x</th>
<th>137x</th>
<th>150x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenastrum capricornutum</td>
<td>7-d Reproduction</td>
<td>3x</td>
<td>45x</td>
<td>156x</td>
<td>238x</td>
<td></td>
</tr>
<tr>
<td>Ceriodaphnia dubia</td>
<td>7-d Growth</td>
<td>50x</td>
<td>200x</td>
<td>321x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pimephales promelas</td>
<td>6-month reproduction</td>
<td>3x</td>
<td>34x</td>
<td>36x</td>
<td>40x</td>
<td></td>
</tr>
</tbody>
</table>

* Effluent contribution could not be differentiated from adjoining tributary.

* Inc = inconclusive; downstream sites differed from some but not all upstream sites.

* Downstream differences for 2 of 16 metrics.

* Mean effluent bioassay 5C25 effect concentration vs. mean instream effluent concentration.
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One aspiration of the LTRWS was to help bridge the information gap between these two approaches with the hopeful target of arriving at an approach that provides for both short-term response indicators of effluent effects as desired by the regulatory community as well as an assurance that these indicators provide an accurate portrayal of the health and sustainability of fish communities over the longer course of time.

Some Career-Based Perspectives

The combined careers of the two authors represent over 65 years of research related to pulp and paper mill effluent effects questions. This long-term period of involvement has provided for the formation of a number of perspectives based both on knowledge gained from the research completed to date as well as a corresponding awareness of future research information needs. Also offered is a nontechnical but nevertheless important view of forests as a unique renewable resource.

The Need for Both Short-Term Indicators of Effect and Long-Term Validation Studies to Address Ecological Relevance

The authors feel great satisfaction in having worked in a field characterized by such dynamic change over the past three-plus decades. This has provided an opportunity not only to carry out challenging and meaningful research, but to also interact with a similarly engaged group of international researchers. There have been, however, frustrations and misunderstandings along the way in terms of the approaches used and resulting conclusions reached regarding effluent effects questions. Part of this may be a result of program-driven differences in research objectives. Regulatory programs, for example, from an environmental protection standpoint, may need to respond proactively to perceived problems or those that are thought to have an unacceptable or high risk. The Canadian EEM program and the use of WET tests in the U.S. NPDES program are examples of the use of proactive short-term or rapid response indicators in safeguarding against possible adverse effluent effects. There is also an interest, however, on the part of the associated industry, that additional process and effluent treatment costs directed toward improvements in effluent quality have a measurable return in terms of benefit to the environment. From this standpoint it is important that the short-term or rapid bioassessment approaches incorporated into environmental protection programs be ecologically relevant in terms of providing for the long-term sustainability of fish populations as well as other components of the aquatic ecosystem. The suggestion here is not that there is a right way or a wrong way to move forward with respect to effluent effects questions, but that both long-term and short-term assessment approaches have merit and those future efforts should not be exclusive to one approach or the other.

The Need for Watershed-Scale Studies

With the advent of effluent secondary treatment and refinements to the mill process, effluent effects have been markedly reduced from much earlier times when incidences of acute toxicity were not uncommon and gross examples of water fouling from fibre deposits and low dissolved oxygen occurred. Today’s pulp and paper mill effluents offer the potential for effluent effects to be assessed at more and more subtle levels. The NCASI LTRWS, for example, provides indication that biological population/community level effects, if they exist, may be below measurable detection limits and that effluent related water quality effects for nutrients and some other parameters may more likely have signatures from nonpoint agricultural or land-use activities than from mill effluent discharges (Hall et al. 2009c). The case is presented here that future studies regarding the effects from pulp and paper mill effluents be carried out at the watershed level scale so that effluent effects can be placed in context with other water quality influencing factors, and so that watershed management and regulatory attention can be directed where the benefit is the greatest.
The Need to Consider a Historical Dimension in Addressing Current Effluent Effect Concerns

The pulp and paper industry is one of the older industries in North America and elsewhere. There are many mills that have been in operation and have been discharging to receiving waters for well over 100 years. Codorus Creek, one of the LTRWS study streams, has actually had a mill in active operation for over 150 years. An argument is not being made that premodern technology mill operations and discharges of untreated effluent were not without adverse effect, but rather, based on Codorus Creek and evidence from the LTRWS, that there has not been a lingering effect or long-term degradation carried forward to modern mill practices and properly treated effluents. This note of optimism is contradicted directly in the print media where dire predictions of adverse effects are sometimes stated. For example, Weinhold (2009) cautions “If you’re reading this on paper, you may want to thank fish populations around the world for their sacrifices,” and that “effluent from pulp and paper mills discharged to nearby waters is linked to plummeting fish populations ...” Even in the absence of contemporary comprehensive studies such as the LTRWS, there should be reason for questioning such dire predictions (e.g., plummeting fish populations), owing the contrary evidence provided from streams which have demonstrated long-term sustainable fish populations in the presence of pulp and paper mill discharges over periods of time spanning many decades.

Some Lingering Effluent Effects May Be Due to the Natural Properties of Wood

A review by Hall and LaFleur (2003) indicated that plants produce several thousand secondary metabolites that provide protection from herbivores. Many of these metabolites are of the high molecular weight water-soluble polyphenol form, which achieve antitherbivore properties through protein binding capacities. Although primarily a defence mechanism against terrestrial herbivores, these same compounds, being water soluble, are also liberated from natural wood residue to surface waters. Concentrations of these materials along with other humic substances produce the strong tea colouration of muskeg, peat bogs, and many coastal and boreal forest streams, as well as pulp and paper mill effluent (Fig. 6). These natural products have been found to adversely affect the colonization settling of various benthic marine organisms, to cause gill damage in fish, and even to function as antimicrobial agents sufficient to be considered for use as a wood preservative or in dental cavity prevention. They may also provide an avenue of explanation for residual bioassay responses at higher effluent concentrations or the fact that reproductive effect indicators noted in the EEM program appear to be unrelated to mill process or treatment type (Dubé et al. 2008; Hewitt et al. 2008). The significance of polyphenols as a factor affecting fish reproduction has also been suggested in the fathead minnow life-cycle work carried out by NCASI (Borton et al. 2009). The possibility exists that residual mill effluent effects may be of similar nature and actually of a much smaller scale compared with the leachate produced by the northern latitude boreal forests.

Forests Are a Unique Renewable Resource

Forests truly represent a unique resource in today’s world where many resources are recognized as being rapidly depleted and nonrenewable in nature. Forest harvest carried out with modern forestry practices provides a sustainable alternative for a wide range of currently important products, including lumber and pulp and paper, and also provides for an important future potential as a cellulose-based energy source. Forests are also being increasingly recognized as a beneficial land use, e.g., “working forests,” achieved through conservation easements and along with farming practices have been recognized as a preferable land use to development (Mapes 2009). The maintenance of timberland, in lieu of deforestation and alternative land uses, may also be essential to the availability of future supplies of high quality water (Wiegand et al. 2009). The remarkable regenerative properties of forests are also in evidence today at the locations of some previous harvest and mill

Fig. 6. A forest leachate coloured coastal tide pool and river in Washington State.
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Fig. 7. Swanson Bay, the location of one of the first pulp mills in British Columbia during mill operations in 1909-1923 (left) and today (right). The stack represents the only visible evidence of the mill site which has now been reclaimed by the forest. (Photo credit: Moore 1974).

operations. A vivid reminder of the renewable nature and resilience of forests is provided by the coastal mill at Swanson Bay in British Columbia (Moore 1974). The mill, one of the first in British Columbia, operated during the period 1909 to 1923. A remarkable reminder of forest resiliency is the single stack which today protrudes through a canopy of naturally regenerated forest at the location of the former mill and its associated town (Fig. 7).

Conclusions

The authors feel gratified in having had long productive careers in a field of rapidly advancing knowledge and continued new challenges. The series of seven “Fate and Effects” conferences has provided evidence of the advances that have been made with respect to effluent effects, and also serves to provide testimony that questions still remain and will continue to be raised in the future. It is clear, however, that the knowledge base regarding effluent effects has advanced considerably since the first conference held in 1991, and that it would be inaccurate to perceive the potential for newly discovered effluent effects to be unlimited, as suggested in the “pulp mill effluent onion” analogy (Hodson 2008). It appears clear that effluents from modern mills with secondary treatment have diminished effects from earlier process and effluent treatment technologies. Long-term studies at the population/community level have, for example, demonstrated a lack of effluent response at multiple trophic levels, and additional bioassays substantiate that this finding comes with a considerable “margin of safety” (Hall et al. 2009c). The indications of diminished effects also come with recognition that some of the residual responses may arise from the natural chemical properties of wood and not as an expression of the pulp or paper making process (Dubé et al. 2008; Hewitt et al. 2008). This finding represents a new challenge in that increasingly subtle effluent effects will require investigation not only with respect to the natural chemical properties of wood represented in effluent, but also how these same properties function naturally in aquatic ecosystems.

Acknowledgements

The authors wish to recognize the late Russell O. Blosser, and Isaiah Gellman, past NCASI vice president and president, respectively, for their efforts in sustaining the vitality of NCASI’s aquatic biology program over many decades.

References


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