Overview of Laboratory Testing for Reproductive Effects of Pulp Mill Effluents in Fish.

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Summary

To assess the effects of individual pulp mill effluents (PMEs), controlled laboratory fish exposures have been conducted. These tests have the benefit that they remove potential confounding effects of historically-contaminated sediments and other contaminant inputs (such as agricultural run off, municipal sewage treatment effluent input, etc.) that may confound interpretation of effects in wild fish collected from the PME receiving environment. Controlled laboratory fish exposures have been used to assess the potential for pulp mill effluents to affect gonad size, reproductive output, or indicators of gonadal growth and function (such as measurement of reproductive steroids or vitellogenin). In the following review, the findings of these short-term and long-term reproductive indicator tests applied to pulp mill effluents are discussed. Short term lab exposures of fish have the advantage of time and cost savings over long term reproductive tests, but their linkage to real effects in wild fish is unknown. Estrogenic compounds in PME will induce Vtg (via binding to the estrogen receptor), but it appears that some PMEs can interference with sex steroids and reproduction of fish at places other than the estrogen receptor. Changes in sex steroid concentrations in lab-exposed fish are more closely linked to effects in wild fish exposed to PMEs. Steroid decreases are often seen in fish captured downstream of PMEs. However, predictive relationships or direct linkages of sex steroid concentrations and gonad size or reproductive potential have not been made. Long-term lifecycle exposures of fish provide some of the most convincing evidence linking PME exposure to reproductive effects in fish to PME. Similar to the effects seen wild fish exposed to PME, lifecycle exposures of small laboratory species have shown growth increases, liver enlargement, alterations in sexual development, delays in time to first spawning, reductions in the numbers of eggs laid, or decreases in levels of sex steroids. It is clear at this time that lifecycle exposures provide the most meaningful and environmentally relevant information of fish exposure to PMEs. However, their widespread use is limited due to the significant time, cost and effort, and difficult logistics of conducting a lifecycle tests. Shortening the lifecycle test or using partial lifecycle tests may prove promising for assessing impact of PMEs on fish.

Keywords: lifecycle test, fathead minnow, vitellogenin, sex steroids, fish bioassay

Short-term fish tests

Vitellogenin induction:

Vitellogenin (Vtg) is an egg protein precursor synthesized by the liver of mature female fish. Synthesis of Vtg in females is governed by estradiol binding to the estrogen receptor. Both female and male fish have the ability to produce Vtg, but it is normally not found in juveniles or males, as levels of circulating endogenous estrogens are normally very low. The presence of elevated concentrations of Vtg in blood of male or juvenile fish can be used to indicate the presence of estrogenic chemicals in effluents. While most of the research assessing Vtg has been examining the impact of municipal waste plant effluents, recent research suggests that some PMEs may be able to elevate Vtg.

Short term exposures of fish to PME have assessed Vtg production and sex steroid concentrations. Juvenile rainbow trout, goldfish, fathead minnow, mummichog, and largemouth bass have been exposed to PMEs and Vtg or sex steroids measured as indicators of potential of the PME to cause reproductive changes.

Three of six pulp mill effluents (tested at 2 % and 20 %) induced Vtg in mature male fathead minnows exposed for 21 d (Martel et al 2004). Vtg was induced by various types of effluent: one kraft mill, one thermomechanical pulp (TMP) mill, and one multiprocess mill. No effects on Vtg were seen with three other effluents from different bleached kraft, TMP and multipocess mills (Martel et al 2004). Recent data from the same lab have shown that 8 of 11 Canadian PMEs have the ability to induce Vtg in rainbow trout and fathead minnows, at exposures up to 20 %. (pers comm., Tibor Kovacs and Pierre Martel, Paprican, Pointe Claire, QC, Canada). Exposure to 1 % final effluent from a BKME induced Vtg in adult male fathead minnows exposed for 21 d (Rickwood et al, 2006).

Other studies and field data have shown suppression of Vtg in female fish after exposure to PMEs. Largemouth bass exposed to ≥ 20 % BKME for 28-56 d had lower Vtg than control females (Sepúlveda et al 2001, 2003). Evidence from male or juvenile fish caged downstream of PME outfalls has often shown no change in Vtg or suppression of Vtg (Mellanen et al 1999; Sherry et al 1999; Karels et al 2001; Svenson et al 2002; Nickle et al. 2003)

The significance of the Vtg induction in male or juvenile fish caused by exposure to some PMEs is being investigated. The observation of Vtg induction by PMEs is relatively new. However, Vtg induction results are sometimes inconsistent with other steroid and reproductive effects seen in wild fish exposed to PMEs. Future research will determine if there is a mechanistic linkage between the PME compounds that cause reproductive effects in fish and those that cause Vtg induction.

Steroid Concentrations:

In an effort to predict reproductive changes in fish exposed to PMEs, tests of fish steroids have been used successfully (McMaster et al., 1996; Parrott et al., 2000; Tremblay and Van Der Kraak 1999; Dubé and MacLatchy, 2000, 2001a, 2001b). It is hypothesized that compounds in PME may

interfere with sex hormone receptors or with key enzymes involved in the synthesis of sex hormones from cholesterol. Interference may also occur at higher levels of hormonal control, at various levels in the hypothalamus-pituitary-gonadal axis.

These short-term fish tests usually showed decreased steroid levels after PME exposure, which mimics the steroid reduction seen in wild fish exposed to some PMEs (reviewed in McMaster et al 2005). However, decreases in steroid concentrations have not yet been linked to meaningful effects on fish reproduction. Physiologically, lower levels of sex steroids produced by the gonads may lead to smaller ovaries and testes, and to negative impacts on fish reproductive capacity. However, among groups of exposed fish, lower levels of sex hormones in individual fish have not been directly related to the size of the gonad in that fish.

After short term exposures (7 to 60-days) to PMEs, goldfish, rainbow trout, mummichog, or fathead minnows have shown alterations in circulating sex steroid concentrations or in sex steroid production by pieces of ovary or testes incubated in vitro (McMaster et al., 1996a; Parrott et al., 2000; Tremblay and Van Der Kraak 1999; Dubé and MacLatchy, 2000a, 2001a, 2001b). In these tests, the sex steroids measured are estradiol, testosterone and 11-ketotestosterone.

Most research has shown that exposure to PME decreased the levels of circulating steroids in fish. Exposure of mature male goldfish to bleached sulphite mill effluent for 21 d caused a drop in testosterone production by goldfish testes to one-seventh of control fish (Parrott et al 2000). Bleached kraft mill effluent (BKME) and TMP mill effluent from Alberta, Canada, also caused decreased in male goldfish circulating testosterone after exposures of only 8 days (McCarthy et al 2003). Mummichog exposed for one month to 1 % primary or 1 % secondary treated BKME reduced plasma T in male and female fish ((Dubé & MacLatchy, 2000). Largemouth bass (*Micropterus salmoides*) exposed for periods of 7 days to 2 months to 10-80 % bleached paper mill effluent had decreased circulating steroids (up to a 40 % decrease in 11-ketotestosterone and 60 % decrease in estradiol of females, and up to a 50 % decrease in 11-ketotestosterone of males, compared to control fish) (Sepúlveda et al., 2001).

Development of techniques for measurement of steroid production by excised gonads of fish (McMaster et al., 1995) allowed the production of steroids to be assessed in testes and ovaries, under either basal or stimulated conditions (with added human chorionic gonadotropin (hCG), to stimulate the gonad to produce hormones). Techniques for measurement of testicular and ovarian in vitro steroid production paved the way for investigations into the effects of PMEs on the reproductive functions of smaller-bodied fish species (too small to sample blood for sex steroid measurement).

Decreases in levels of sex steroid production by excised gonads are seen in some fish exposed to PMEs. Marine mummichogs exposed to PMEs showed decreases in production of sex steroids by excised testes and ovaries (Dubé & MacLatchy, 2000). In general, measurements of steroids produced by excised gonads correlate well with levels of circulating steroids.

Short-term exposures of fish to PME and assessment of sex steroids has shown similar responses as seen in wild fish captured from PME receiving environments. The potential linkage of depressed steroids with smaller gonads can be made mechanistically, but the cause-effect relationship has not been proven.

Long-term fish tests

Long term exposures of fish to PME have shown changes in egg production, secondary sex characteristics, gonadosomatic index (GSI), liver-somatic index (LSI), and growth. In North America, fish lifecycle tests have usually been conducted with fathead minnows. Lifecycle exposures require several months, and large quantities of effluent. However, they are the definitive test for assessment of PME in the lab, and integrate responses of fish over time, with exposures of all stages of the fish lifecycle (OECD 2000a, 2000b; Parrott et al 2001). Because growth, development and reproduction can be measured, these tests provide some of the closest links to the effects of PME exposure on wild fish.

Fathead Minnow Lifecycle Studies

Many types of PMEs have been evaluated using fathead minnow lifecycle tests. Most PME have the ability to reduce reproduction in fathead minnows. Robinson (1994) found that exposure to 12.5 % BKME reduced reproduction. Borton et al (2001) evaluated effluent from eleven pulp mills: eight kraft mills (two of which had no bleaching, two with Cl bleaching, one with elemental-chlorine-free (ECF) bleaching, and two with oxygen delignification (OD) and 70 % CLO_2 bleaching), two re-cycled fibre furnish mills, and one thermomechanical mill. No effects were found in fish exposed for a lifecycle to effluent from the thermomechanical pulp mill and from one of the recycled fibre mills (that used de-inked office paper as furnish) up to 100 % effluent. All other (nine of eleven) mill effluents significantly decreased fish reproduction, with effective concentrations for 25 % reduction in reproduction (EC25s) ranging from 17 % to > 100 % final effluent. Final effluent from an unbleached mill reduced reproduction in fathead minnows (EC25 = 38 %) and delayed reproduction by 12 days (at the EC25 effluent concentration) (Borton et al 1997, 2001).

Kovacs et al have performed three lifecycle studies with PMEs. Effluent from a thermomechanical mill had no effects on fathead minnows when tested at concentrations up to 20 %. Exposure to BKME significantly affected fish reproduction and sex characteristics at 2.5 and 5 % effluent (Kovacs et al 1995a). This effect was not seen in similar lifecycle tests performed 2 years later after changes were made to the BKME process and treatment (Kovacs et al 1996).

Bleached sulphite mill effluent (Parrott et al 2004) and several other mill effluents (Borton et al 2000) have been tested using fathead minnow lifecycle assays conducted in situ in a bioassay trailer at the mill secondary treatment ponds. This allowed the assessment of fresh effluent rather than effluent that had been batch-collected every 1-2 weeks and shipped to laboratories for fish exposures.

Specific Endpoints

Some endpoints in lifecycle tests are not very sensitive to the effects of PMEs. Hatching success of fathead minnow eggs exposed to PMEs is not a very sensitive endpoint or predictive

indicator of effects, and concentrations that affect hatch are usually several-fold above concentrations that cause decreases in reproduction. If egg hatch is affected, the effluent is usually overtly toxic, and 7-d fathead minnow acute tests may confirm this.

Growth can be increased or decreased in lifecycle tests of fathead minnows exposed to PMEs. Decreased growth was seen in fathead minnows exposed to high concentrations of BKME (NCASI 1985; Robinson 1994). Most studies have reported increased growth of fathead minnows exposed to PMEs. Female fathead minnows exposed to 100 % BKME for a lifecycle (178 d) were 46 % and 92 % heavier than control females, and males from 100 % BKME were 44 % heavier than control males (NCASI 2000). Similarly, length and weight of female fish was greater in bleached sulphite mill effluent concentrations of 10 % and above, with a doubling of fish weight in fish exposed to 30% effluent compared to control fish (Parrott et al 2003, 2004).

Decreases in the production of gonadal sex steroids is also seen with lifecycle exposure to PMEs. In most cases concentrations of sex hormones in blood and the production of sex hormones by excised testes and ovaries are less sensitive indicators of effect than reproductive output (Robinson 1994; Borton et al. 1997; NCASI 2000).

Changes in sexual development and decreases in or delays in the expression of secondary sex characteristics have been seen in lifecycle exposures of fish to PMEs. Delayed development of sex characteristics and de-masculinization of male fish have been seen in fathead minnows exposed to BKME (Robinson, 1994; NCASI 2000). In one case the concentration of BKME (12 %) that reduced sex characteristics was lower than that needed to cause significant decreases in the time to first spawn and the number of spawns (NCASI 2000).

Masculinization of female fish and a shift of sex ratios towards more male fish have been seen after exposure of fathead minnows to PMEs. Fathead minnows exposed to concentrations of 2.5 % secondary-treated BKME and above for 275 d had increased male secondary sexual characteristics (Kovacs et al., 1995a). Masculinization has been seen in adult female mosquitofish (Ellis et al., 2001) sticklebacks (*Gasterosteus aculeatus*) (Katsiadaki et al., 2002) and guppies (Larsson et al., 2002) exposed to PMEs.

Feminization of male fish has also been seen in lifecycle exposures of fathead minnows to pulp and paper mill effluents. Fathead minnows exposed to BSM effluent from a Canadian mill for 5 months showed an increasing proportion of fish with ovaries, premature development of ovipositors and development of ovipositors in mature male fathead minnows (Parrott et al., 2003, 2004).

The liver-somatic index (LSI) may be increased in fathead minnows exposed to PMEs. Enlarged livers usually occur in fathead minnows exposed to high PME concentrations (greater than 50 % effluent). Increases in LSI usually require higher PME-exposure concentrations than those required to reduce fathead minnow reproduction (Borton et al 1997; NCASI, 2000; Parrott et al 2004).

Gonadosomatic indices **(**GSIs) can be reduced or increased by exposure of fathead minnow to PMEs, but impacts on GSI occur at higher concentrations than impacts on reproduction (Borton et al 1997, 2004, Parrott et al 2004). Exposure to 10 % BSME for a lifecycle reduced fathead minnow egg production to one-eighth of control fish egg production, even though female and male gonad sizes were not significantly different from the control fish (Parrott et al 2004).

Reproduction

Reproductive success of fathead minnows exposed to PMEs can be measured as age at first spawn, total number of eggs produced, number of eggs per female, or number of eggs per female per day. This endpoint may be the most physiologically- and environmentally-relevant change seen after PME exposures of fish. In addition, most studies show that for long-term or lifecycle exposures, it is the most sensitive response seen.

Lifecycle exposures of fathead minnows to PMEs have usually found that egg production and time to first spawning are the most sensitive parameters studied (NACSI 1985, 1996; Robinson, 1994; Kovacs et al., 1995a; Borton et al., 1997, 2000; Parrott et al., 2003, 2004). Effects of PME on fish reproduction are dramatic. One of the earliest reported pulp mill fathead minnow lifecycle tests assessed secondary treated effluent from a bleached kraft mill (Robinson, 1994). After 6 months' exposure to 3 to 50 % BKME, fathead minnows had significantly reduced egg production and significantly delayed spawning compared to control fish. Fathead minnows exposed to four pulp mill effluents had average spawning delays of 10 to 14 days at the EC25 effluent concentration (Borton et al 1997). Egg production (measured as number of eggs per female per day) correlated most strongly (R^2=0.65) to time to first spawning, in a study of five lifecycle tests of four different PMEs (Borton et al., 1997).

Fathead minnows exposed to bleached sulphite mill effluent for a lifecycle had an 80% decrease in egg production at 10 % effluent. A surprising finding was that female GSIs were similar to control fish GSI, even though egg production was so much lower than controls (Parrott et al. 2004). Effects on egg production are often dramatic: Fish exposed to 100 % BKME produced 0.1 egg/female/day, and exposure to 50 % BKME resulted in egg production of 6.8 eggs/female/day (compared to 14.5 eggs/female/day in control fish) (NCASI 2000).

Although lifecycle fish tests provide relevant information about exposure of fish to PMEs, there are several limitations to their use. The fathead minnow lifecycle test is lengthy, and logistically difficult. A fathead minnow lifecycle test is expensive, and requires at least 2 people for 5 months. Effluent exposure requires either setting up the test in situ, or shipping large quantities of effluent (600 to 5,000 L/week, depending on concentrations of effluent tested) (Kovacs et al 1995a, 1995b, 1996; NCASI 2000; Parrott et al., 2003, 2004). Because the test is meaningful, but costly, there are several attempts to shorten or modify the fathead minnow lifecycle test while still retaining the biologically-relevant endpoints.

Shorter Tests of Fish Reproduction

The common finding of reproduction as the most sensitive response in fish exposed for a lifecycle to PME and the observation that cessation of reproduction in PME-exposed adult fish can be a sensitive and immediate response (Borton et al 2004) holds promise for shortening the lifecycle tests, while still maintaining this important and meaningful endpoint.

The fathead minnow adult terminal reproductive assay, outlined in Harries et al (2000) and Ankley et al. (2001) has recently been tested with PMEs. The test monitors breeding success (egg production, secondary sex characteristics) of mature fathead minnows in control water for 3 weeks. Then, exposures of PME begin, and breeding and sex characteristics are tracked, and fish are sampled to measure bioindicators such as vitellogenin, sex steroids, and gonad histopathology.

Cessation in egg production in mature, breeding fathead minnows was seen in only one of the six PMEs (at 20 % dilution, for 4 weeks) (Martel et al 2004). However, when reproductive biomarkers were examined in the PME-exposed fish, nearly all of the effluents had some effect. Five of the six PMEs significantly affected one (or more) of the reproductive indicators: whole body sex steroids, male secondary sex characteristics, and vitellogenin (Martel et al. 2004). Fathead minnows exposed to 50 % and 100 % BKME for 3 weeks had decreased egg production (Rickwood et al., 2003), and hatching success of eggs in 100 % BKME was reduced to 15 % (from 80-90 % hatch in control eggs) (Rickwood et al., 2003). As well, alterations in mature adult secondary sex characteristics can be seen in exposures as short as 21-d: Mature male fathead minnows exposed to BKME for 21-d showed development of ovipositors (Rickwood et al 2006).

The shorter test is also potentially useful for investigation of cause experiments, assessing specific pulp mill waste streams for ability to affect fish reproduction. Exposure for 21 d to 5.75 % combined alkaline sewer effluent from a BKME reduced cumulative egg production in fathead minnow (Rickwood et al, 2006),

Predictive Ability of Fish Bioassays

Short-term lab exposures of fish have the advantage of time and cost savings over long-term reproductive tests, but their linkage to real effects in wild fish exposed to PMEs is unknown for Vtg and hypothesized for sex steroids. Estrogenic compounds in PME will induce Vtg via binding to the estrogen receptor, but it also appears that some PMEs interfere with sex steroids at places other than the estrogen receptor. As well, fish reproduction is a complex interplay of hormonal and environmental factors that are impacted at many levels other than the estrogen receptor.

Changes in sex steroid concentrations in lab-exposed fish are more closely linked to effects in wild fish exposed to PMEs. Steroid decreases are often seen in fish captured downstream of PMEs. However, predictive relationships or direct linkages of sex steroid concentrations and gonad size or reproductive potential have not been made.

Lifecycle assays were able to mimic many of the responses of wild fish exposed to PMEs. Increased body size (length and weight) and increased liver size are seen in fish after lifecycle and long-term exposures to PMEs. Enlarged livers are seen in both fathead minnows exposed for a lifecycle to PMEs in the lab and in fish captured at sites downstream of PME discharges (reviewed in McMaster et al., 2005).

Decreases in gonad weight and increases in liver weight, condition factor, and age were the most common response pattern seen in the EEM surveys of wild fish exposed to Canadian PMEs (Environment Canada 2002, Lowell et al. 2003, 2004). This pattern of effects in wild fish exposed to PMEs was classified as ‘a form of metabolic disruption in combination with a nutrient enrichment effect’ (Lowell et al., 2003, 2004).

Long-term lifecycle exposures of fish provide some of the most convincing evidence linking PME exposure to reproductive effects. Lifecycle tests of fathead minnows have examined growth, maturation and reproduction. Similar to the effects seen wild fish exposed to PME, lifecycle exposures of small fish species in the laboratory have shown growth increases, liver enlargement, alterations in sexual development, delays in time to first spawning, reductions in the numbers of eggs laid, or decreases in levels of sex steroids. It is clear at this time that lifecycle exposures provide the most

meaningful and environmentally relevant information of fish exposure to PMEs. However, their widespread use is limited due to the significant time, cost and effort, and difficult logistics of conducting a lifecycle tests.

As lifecycle exposures and partial lifecycle tests or adult terminal reproductive tests are conducted with more PMEs, we will be able to asses the predictive ability of these tests, and linkages to health effects in wild fish exposed to PMEs. As more of these tests are conducted with the same effluents, linkages of reproductive effects and biomarkers of effect (such as Vtg and sex steroids) will be possible. It is hoped that these linkages will lead to the development and selection of a (suite of) fish test(s) that can predict the impact of PME to fish, in shorter timeframes, but without loss of biological or environmental relevance.

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