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Impacts of Fenholloway River Water on Invertebrate Biodiversity and Growth of *Daphnia magna*

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The Fenholloway River is located entirely within Taylor County along the northern Gulf coast of Florida (Fig. 1). A pulp mill has released Pulp Kraft Mill Effluent (PKME) into the stream just outside of Perry, FL. Recent studies conducted near the mill have demonstrated that female Eastern mosquitofish, *Gambusia affinis holbrooki*, in the Fenholloway River have a gonopodium-like fin present, indicating that they have been masculinized by an androgenic endocrine-disrupting compound (Parks et al. 2001). The gonopodium is a modification of anal fin rays 3, 4, and 5 that is used to transfer sperm from males to females. Based upon the number of segments present in the longest anal fin ray, data indicates that 80% of the female mosquitofish have been partially masculinized and 10% have been fully masculinized. In vitro, Fenholloway River PKME shows affinity for the human androgen receptor (Parks et al. 2001).

Endocrine disrupting effects of Fenholloway River water on invertebrates has not been assessed, nor has ecosystem health been reported. Daphnids are extensively used to assess effluent toxicity in the U.S., and the effects of endocrine disruptors including ecdysteroids, estrogens and androgens have been determined in *Daphnia magna* (Baldwin et al. 1995; 2001; Olmstead and LeBlanc 2000). For example, Diethylstilbestrol (DES) and androstenedione decreased overall growth of daphnids 20-25% at 3µM and 25µM, respectively (Olmstead and LeBlanc 2000), and DES also decreased fecundity in the 2nd generation (Baldwin et al. 1995). Furthermore, plant sterols from PKME have been shown to cause masculinization in the mosquitofish *Gambusia affinis affinis* (Denton et al. 1985). The arthropod steroid hormones (20-hydroxyecdsone, Ponasterone A) involved in molting (Chang 1993) have been isolated from plant material (Nakanishi 1992), and therefore may potentially be in PKME (Slama and Williams 1966). *Daphnia magna* exposed to 260n*M* 20-hydroxyecdysone or 27n*M* ponasterone A exhibited incomplete ecdysis, a lethal incomplete excavation of the molt (Baldwin et al. 2001). Therefore, *Daphnia magna* were treated in 2-generation life-cycle toxicity tests and examined for changes consistent with endocrine disruption.

The PKME may also have significant ecological effects that reduce macroinvertebrate biodiversity (Mayack and Waterhouse 1983). Invertebrate

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biodiversity is considered an excellent biomarker of the health of an aquatic ecosystem (Barbour et al. 1999). Therefore, macroinvertebrate biodiversity, along with some basic water quality parameters were determined and compared to nearby Spring Creek to establish whether PKME effluent from Fenholloway River is decreasing macroinvertebrate biodiversity, and causing endocrine disrupting effects in *Daphnia magna*.

MATERIALS AND METHODS

The Fenholloway River and Spring Creek are third-order streams flowing in a generally southwesterly direction through Taylor County, Florida and emptying into the Gulf of Mexico. Riparian vegetation in the region of the collection stations can generally be characterized as second-growth upland and wetland hardwood hammocks. The substrate is typically sandy with some silt and most commonly covered by no more than 2 meters of water. The average depth at the sampling sites was one meter or less, and moderate flow rates of 0.3 - 0.5 m/s were observed. Two collection sites are described below and their locations relative to the source of paper mill effluent are shown in Figure 1. Site #1: Reference site located at US98 overpass on Spring Creek west of Perry. This site is near the Fenholloway River, and has similar width and water depth compared to the site on the Fenholloway River below the mill outfall. Site #2: Located near the US 19/98 overpass on the Fenholloway River and downstream of the mill outfall. Stream width, depth, flow rate and substrate were similar to Site #1, except substrate contained more silt. Water was collected slightly upstream from site #2 to obtain water closer to the effluent discharge. A site upstream of the mill was not analyzed as a reference site because the Fenholloway River downstream of the mill is much wider and contains primarily effluent.

Water was collected from sites 1 and 2 on May 9, 2001, by placing 24 new one-liter, amber capped bottles below the water surface, and then unscrewing the cap underwater for filling of the water bottle. The cap was screwed back on the bottle underwater after it was filled. The water was stored on ice in coolers until returned to the lab where it was refrigerated at 4°C for the duration of the study. Water was not collected on July 20, 2001.

Water quality parameters were measured on May 9, 2001 and July 20, 2001. Salinity, conductivity, and temperature were determined with a YSI Model 30 portable SCT meter (Yellow Springs Instruments, Yellow Springs, OH). A YSI Model 55 portable dissolved oxygen meter (Yellow Springs Instruments, Yellow Springs, OH) was used to measure the dissolved oxygen. Turbidity was measured using a HACH Pocket Turbidimeter (HACH Co., Loveland, CO). pH was determined using a pH meter after transport of water back to the laboratory.

Benthic invertebrates were collected at both sites during spring (May 9, 2001) and summer (July 20, 2001) to determine macroinvertebrate biodiversity in the Fenholloway River. Macroinvertebrates were collected according to EPA protocol

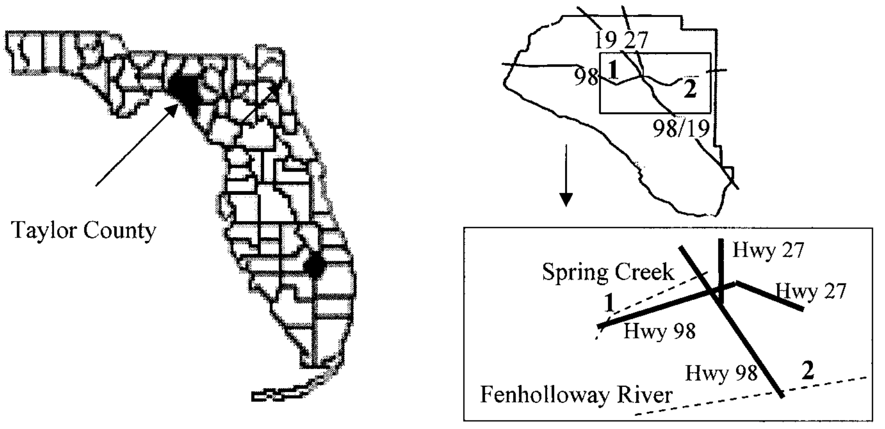

Figure 1. The Fenholloway River (site 2) is located in Taylor County, FL. A reference site was examined (site 1) at Spring Creek west of Perry, FL. Rivers are represented by dashed lines and roads by solid lines.

standards (Barbour et al. 1999), using a rectangular dipnet to sample along the bank vegetation of the streams. Approximately 200-350 specimens were collected at each site, placed in 80% ethanol and transported to the laboratory for identification with the aid of keys in Merritt and Cummins (1996) and counted for use in several diversity indices including taxa richness, composition measurements, and tolerance/intolerance measures (Barbour et al. 1999). Statistical differences in biodiversity were determined using Fisher's exact test.

Female *Daphnia magna* were exposed to effluent water collected on May 9th through two generations in static renewal chronic toxicity tests according to previously published methods. Each generation exposure lasted 21 days. The first generation exposure consisted of 3 exposure treatments, with 10 daphnids per treatment. Female daphnids less than 24 hours old were housed at 25°C ± 1°C in a 16:8 light:dark photoperiod individually in 50mL beakers containing 40mL of either moderately hard water (91 $\pm$ 5 mg/L $CaCO_3$)(USEPA, 1993), Spring Creek water, or Fenholloway River water. Spring Creek water was adjusted to pH 7.0 to mimic the pH conditions of the Fenholloway River. Daphnids were fed Red Star® active dry yeast, trout chow from USEPA Duluth (YT) and *Selenastrum capricornium,* (4 X 10^6 cells per day until 7 days of age and 8 X 10^6 cells per day thereafter) which was cultured in Fritz f/2® algae medium (Baldwin et al. 2001). Water was changed three times a week, at which time molts and offspring were counted. At 21 days offspring less than 24 hours old were randomly selected for second-generation studies using moderately hard water, Spring Creek water and Fenholloway River water. Static renewal chronic toxicity tests were conducted with the second generation in the same manner as the first generation studies. Statistical differences were analyzed by ANOVA. The body length of daphnids at the end of each of the first and second generation studies were measured as defined by Olmstead and LeBlanc (2000) using a micrometer under a microscope magnified 10X. Statistical differences between the lengths of control and exposed daphnids were determined by ANOVA.

RESULTS AND DISCUSSION

Table 1 shows water quality data taken on May 9 and July 20, 2001 from Spring Creek and the Fenholloway River. The Fenholloway River downstream from the mill had lower dissolved oxygen and higher turbidity, water temperature, salinity and conductivity than Spring Creek. Dissolved oxygen measurements dropped as measurements were taken further downstream of the mill (data not shown), indicating organic pollution.

Macroinvertebrates were collected at sites 1 (Spring Creek) and 2 (Fenholloway) (Fig. 1) to determine biodiversity downstream of the mill compared to Spring Creek. Nineteen distinct families were collected from the reference site while only eight families were collected from the Fenholloway River. Most of the invertebrates collected from the Fenholloway River are considered tolerant, while Spring Creek contained both tolerant and intolerant invertebrates (Barbour et al. 1999). Invertebrates collected at Spring Creek include Palamonidae, Camboridae, Anisoptera-Gomphidae, Anisoptera-Libellulidae, Zygoptera-Coenagrionidae, Amphipoda, Ephemoptera-Tricorythidae, Ephemoptera-Heptageniidae, Ephemoptera-Ephemerillidae, Ephemoptera-Baetidae, Ephemoptera-Baetiscidae, Coleoptera-species, Coleoptera-Haliplidae, Coleoptera-Dryopidae, Gastropoda-Planorbidae, Gastropoda-Physidae, Gastropoda-Pilidae, Bivalvia-Corbiculidae, Chironomidae, Hemiptera-Gerridae. Invertebrates collected at Fenholloway were Gastropoda-Physidae, Chironomidae, Hemiptera-Belostomatidae, Coleoptera-Haliplidae, Coleoptera-Dytisidae, Zygoptera-Coenagrionidae, Anisoptera-Libellulidae, and Amphipoda.

Several other metrics also indicated that the Fenholloway River downstream of the mill demonstrated reduced biodiversity. Table 2 displays a variety of metrics calculated from the two sampling dates during two seasons. All of these metrics are indicators of the diversity and dominance within a site (Barbour et al. 1999). All biodiversity indices examined demonstrated that significant reductions in biodiversity were apparent in the Fenholloway River when compared with Spring Creek, including no pollution sensitive species in the Fenholloway River, and the river demonstrated significant dominance by one taxa.

Mayack and Waterhouse (1983) noted that species richness, density, evenness, and diversity were negatively correlated with the physical parameters of turbidity, suspended and substrate-deposited particulates in their study of benthic macroinvertebrates and paper mill effluent in New York State. Concentrations of dissolved oxygen of less than 3mg/L are usually thought to be stressful to most aquatic organisms (Lind, 1985). The high numbers of chironomids found at impacted site 2 are consistent with higher sediment loads, organic pollution and

Table 1. Water quality parameters examined during water and macroinvertebrate collection near Perry, FL on May 9 and July 20, 2001.

	May 9		July 20	
Water Quality Parameter	site 1	site 2	site 1	site 2
Dissolved Oxygen (mg/L)	6.21	5.02	5.25	1.93
Turbidity (NTU)	3.3	20	16.6	29.4
Water Temperature (^{o}C)	19.9	27.8	22.6	30.7
Salinity (ppt)	0.2	1.4	0.2	1.3
Conductivity ($\mu S/cm^2$)	400	2750	464	2544
pH	7.75	6.94	n.m.	n.m.
Hardness	224	300	n.m.	n.m.
Alkalinity	180	96	n.m.	n.m.
GPS coordinates (N)	30.05.037	30.03.942		
GPS coordinates (W)	83.28.326	83.39.854		

The sites are outlined in the methods and materials.
n.m.: not measured
NTU: nephlometric turbidity units
ppt: parts per thousand
$\mu S/cm^2$: microSiemens per centimeter squared

lower dissolved oxygen concentrations seen at this site compared with Spring Creek. Likewise the absence of corbiculids at site 2 and their presence at site 1 might be expected considering their filtering mode of feeding (Pennak, 1989). The Gomphidae are generally burrowers in mud, sand, or light silt (Merritt and Cummins, 1984). For most members of this family deep silt and mud would seem to be detrimental. More gomphids were noticed at site 1 than site 2. Members of the Coenagrionidae are climbers on vegetation (Merritt and Cummins, eds., 1984). Siltation and increased turbidity would likely not affect them as negatively as the burrowers. More coenagrionids were observed in the Fenholloway than in Spring Creek. Overall, the various indices of biodiversity (Table 2) demonstrate reduced macroinvertebrate biodiversity in the Fenholloway River compared to Spring Creek.

Water from Spring Creek, the Fenholloway River and moderately hard water prepared in the laboratory were used to conduct 2-generation life-cycle tests with daphnids, and biomarkers of endocrine disruption were observed. Daphnids exposed to Spring Creek, the Fenholloway River and moderately hard water for both first and second-generation chronic toxicity tests did not show statistical differences in survival, production of offspring, incomplete ecdysis or molting (data not shown). This indicates that the pulp mill effluent did not disrupt reproduction, nor did it effect molting or cause incomplete ecdysis, an indicator of ecdysteroid exposure (Baldwin et al. 2001). Since incomplete ecdysis was not observed in daphnids exposed to Fenholloway River water, this suggests that ecdysteroids are either not present in the pulp effluent or are not at sufficient concentrations to adversely affect the daphnids.

Table 2. Macroinvertebrate sampling results in the Fenholloway River and Spring Creek on May 9 and July 20, 2001.

	May 9, 2001			**July 20, 2001**		
Indicies of Biodiversity	Site 1	Site 3	p-value	Site 1	Site 3	p-value
Families present[a]	17	5		12	7	
% EPT families[b]	23.5	0	<0.0001	16.6	0	<0.0001
% EPT Organisms[c]	17.8	0	<0.0001	4.9	0	<0.0049
% Snails + Mussels	34.2	0.36	<0.0001	49.5	0.4	<0.0001
% Dominant Taxa[e]	19.1	92.1	<0.0001	35.5	88.7	<0.0001
% Tolerant Organisms[f]	35.2	97.8	<0.0001	58.0	94.6	<0.0001
Florida Index[g]	22	0		14	0	
Community overlap - Horn Index[i]		0.158			0.346	

Various indices of biodiversity stream health were used to assess whether the Fenholloway River (site 2) demonstrates significant ecological stress and altered biodiversity compared to Spring Creek (site 1), a local Creek with similar vegetation, water flow and width. Statistics were performed using Fisher's exact test (2x2) with coded raw data.

[a]Total number of families present at a site: Higher number indicates greater biodiversity.

[b]Number of sensitive EPT families (Ephemeroptera, Plecoptera, or Trichoptera) compared to the total number of macroinvertebrate families: A high percentage of EPT families may indicate a relatively unpolluted site.

[c]Number of pollution sensitive EPT organisms compared to the total number of macroinvertebrates from all taxa: A high percentage of EPT organisms may indicate a relatively unpolluted site.

[d]percentage of snails and mussels at the site. These organisms are considered pollution sensitive and a high percentage may indicate a relatively unpolluted site.

[e]percentage of organisms in the dominant taxa: A high percentage indicates low biodiversity and thus may indicate high pollution.

[f]A high percentage of tolerant organisms indicates that sensitive species may be unable to reside in the stream site.

[g]Florida index is a weighted sum of intolerant taxa, which are classified as 1 (least tolerant) or 2 (intolerant). Florida index = 2X class 1 + class 2 taxa. A higher number indicates high biodiversity and typically a healthy stream.

[i]Horn index of community overlap (R_0) is 0 when two communities have no species in common and is a maximum of 1.0 when the species and relative abundances are identical in both communities (Horn, 1966)

Daphnids were also measured following the chronic toxicity tests because androgens have been shown to alter growth (Olmstead and LeBlanc 2000). The Fenholloway River water did not cause a statistically significant change in body lengths relative to Spring Creek and moderately hard water in the first generation. In contrast, there was a statistically significant reduction in size of the daphnids following second-generation exposure to Fenholloway River water compared to Spring Creek (Table 3). Water was stored for effluent tests for up to 42 days. It is possible that the effects observed in the daphnids exposed to Fenholloway

Table 3. Examination of reproductive, molting and growth parameters in female *Daphnia magna* during 21-day chronic toxicity tests with first (a) and second generation (b) *Daphnia magna* exposed to water from site 1 and site 2.

a

Exposure Water	Births[a]	Molts[b]	Body Length (mm)[c]
Moderately Hard	200 ± 15	12 ± 1	5.40 ± 0.19
Site 1 (Spring Creek)	202 ± 34	12 ± 2	5.16 ± 0.26
Site 2 (Fenholloway)	210 ± 46	12 ± 1	5.04 ± 0.44

b

Exposure Water	Births[a]	Molts[b]	Body Length (mm)[c]
Moderately Hard	153 ± 27	12 ± 0.5	5.16 ± 0.20
Site 1 (Spring Creek)	195 ± 13	12 ± 1	5.54 ± 0.14
Site 2 (Fenholloway)	180 ± 25	12 ± 1	4.86 ± 0.34*

Data is expressed as mean ± standard deviation.
Data was analyzed by ANOVA followed by Scheffe's multiple comparison test. An asterisk indicates statistical significance ($p < 0.05$)
[a]Number of living offspring released per female *Daphnia*.
[b]The average number of molts produced over 21 days.
[c]Length of a 21 day old *Daphnia* body under 10X magnification as defined by Olmstead and LeBlanc (2000).

River water were due to reduced water quality during storage that occurred at a faster rate in the Fenholloway River than the Spring Creek water. It is also possible that during storage at 4°C a highly volatile endocrine disrupter evaporated and therefore few effects were observed. However, endocrine disrupting effects on growth and molting of daphnids have been observed in 3-6 days (Baldwin et al. 1995; Olmstead and LeBlanc, 2000), and neither growth nor molting effects were observed in the first generation. Since survival and reproduction were not affected, the ecological relevance of reduced growth is questionable. Biomass can also be affected by other parameters, indicate stress on an ecosystem or organism, and suggest energy utilization is needed for survival and not growth. Reduced size may affect the ability of future generations to produce a high number of offspring, but it did not affect the second-generation Fenholloway River-exposed daphnids. Ultimately, the daphnid life-cycle tests did not indicate that the PKME would seriously reduce the ecological health of the river. However, invertebrate biodiversity was significantly perturbed (Table 2).

In conclusion, the effect of Fenholloway River water on invertebrates was investigated because of the masculinization of female mosquitofish. We have demonstrated that the Fenholloway River contains substances that significantly reduce invertebrate biodiversity, and weakly diminish daphnid growth, thus demonstrating adverse effects on invertebrates in the Fenholloway River in addition to those previously shown in vertebrates (Parks et al. 2001).

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