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Effects of Industrial and Domestic Wastewaters on Selected Biological Indicators in Aquatic Organisms

C. R. Bankston, K. N. Baer

School of Pharmacy, Department of Toxicology, The University of Louisiana at Monroe, Monroe, LA 71209-0470, USA

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The modulation of biological indicators in fish following exposure to industrial or domestic wastewater streams has recently gained the attention of environmental health professionals. Traditional bioassays employing fathead minnows and invertebrates such as *Ceriodaphnia*, focus on larvae survival and reproduction, respectively. These studies are required by the National Pollution Discharge and Elimination Systems (NPDES) permitting process to control the discharge of pollutants into surface waters by industrial or domestic point sources. However, the endpoints utilized in these traditional bioassays may not reveal subtle physiological and/or biochemical alterations resulting from long-term exposure. As a result, they may not be predictive of the environmental impact of wastewater discharges into aquatic ecosystems. Current research has focused on biological indicators of endocrine disruption and, to a lesser extent, the immune system to assess the environmental impact of wastewater discharges.

A variety of effluents or contaminated surface waters have been demonstrated to modulate biological indicators in freshwater fish. For example, fathead minnows (*Pimephales promelas*) exposed to treated municipal sewage effluent resulted in an elevation in vitellogenin (VTG) and a decrease in gonadosomatic index (GSI), hematocrit, and condition factor (Hemming et al. 2001). Surface water from a pesticide-contaminated lake decreased sperm count and impaired sexual behavior in mosquitofish (*Gambusia holbrooki*) (Toft and Guillette 2005). Pulp and paper mill effluent reduced several immunological endpoints (lymphoid organ wt and plaque-forming cell assay) in *Channa punctatus* (Bloch) (Fatima et al. 2001). These endocrine and immunological indicators of contaminant stress are currently not required in traditional bioassays.

The purpose of the present study was to evaluate selected biological endpoints in common carp (*Cyprinus carpio*) following a 42-d exposure to domestic sewage treatment effluent, municipal landfill leachate, and surface water collected downstream from a pulp and paper mill effluent discharge. These preliminary results were compared to the traditional 7-d fathead minnow embryo-larval assay and *Ceriodaphnia dubia* survival and reproduction assay. The results may contribute to the assessment of the environmental impact of wastewaters.

Correspondence to: K. N. Baer

MATERIALS AND METHODS

Juvenile common carp (*Cyprinus carpio*, less than 90 d old) were purchased from Aquatic Research Organisms (Hampton, NH) and acclimated for approximately 1 yr in 520 L Min-O-Cool holding tanks supplied with dechlorinated tap water. During the acclimation period, the temperature was held at 24 ± 2°C and the dissolved oxygen level never dropped below 6.0 mg/L. A 16-hr light/8-hr dark photoperiod was automatically maintained with cool-white fluorescent lights (approximately 200 lux). Transitional lighting was not employed. Fish were fed New Life Spectrum large fish formula pellets (New Life International, Inc.) twice daily on weekdays and once daily on weekends. Mortality during the acclimation period was less than 1%. Fathead minnow (*Pimephales promelas*) embryos (<24 hr old) and gravid *Ceriodaphnia dubia* adults were purchased from Aquatic Research Organisms. Fathead minnow embryos were used immediately and *Ceriodaphnia* neonates (less than 24 hr old) were collected from adult cultures the following day.

Grab samples from three sites in northeast Louisiana were collected at regular intervals from June 14, 2004 to the end of the study (42 d). The three sites were a domestic sewage treatment effluent (ST), municipal landfill leachate (LL), and surface water collected approximately 3.5 km from a pulp and paper mill effluent discharge (PM). Prior to testing, 48-hr acute tests employing *Ceriodaphnia dubia* neonates were conducted on all undiluted wastewaters. No mortality was observed with ST and PM samples, but an LC_{50} of 18% was observed with the LL samples (results not presented). Therefore, the landfill leachate was diluted to 10% with laboratory water and used for testing. During the sampling period, daily air temperatures ranged from 17 to 37°C and weekly rainfall ranged from 0.10 to 8.76 cm with an average of 4.26 cm/wk. Water samples were collected into 20-L polycarbonate containers and either used immediately or stored at 4°C (<72 hr) until use.

For the carp study, exposure vessels were 38 L glass aquaria containing approximately 30 L of testing solution. There were two replicates per treatment with 8 fish per replicate (total 16 fish per treatment; control loading was 0.36 g/L). Fish were added to replicate tanks by random number assignment. Control replicates contained dechlorinated laboratory tap water. Due to low dissolved oxygen levels in PM samples, all wastewater samples were aerated continuously during the exposure period. Replicate tanks were renewed three times weekly (Monday, Wednesday, and Friday) with either freshly collected samples or warmed samples previously stored at 4°C (<72 hr). Animals were fed large fish pellets twice daily on weekdays and once daily on weekends (approximately 1 g per feeding per replicate tank). During renewals, water was removed by siphoning (to an approximate 2 in. depth) and fresh solution gently added. Feces and debris were also removed during renewals. Temperature, dissolved oxygen, pH, conductivity, and total hardness were measured in selected replicates (both old and new solutions) at least twice weekly. The fathead minnow and *Ceriodaphnia dubia* 7-d studies were conducted using samples collected during

days 10 through 17. These studies were conducted according to USEPA testing guidelines (USEPA 1994).

After the 42-d exposure, carp were sacrificed by pithing and standard length (cm), total body wt (g), liver, spleen, and gonad wt (g) were recorded. Condition factor (wt x 10^5/length^3) and hematocrit (packed blood cell column height/total blood column height x 100) were calculated for individual fish to assess fish health. Liver-, spleen-, and gonadosomatic index (respective organ wt x 100/total body wt) were calculated to assess the physiological effects of exposure. Male/female ratios in controls, 10% LU, STP, and PM tanks (determined following sacrifice) were 4/12, 8/8, 9/7, and 9/7, respectively.

Blood sampling was conducted by caudal transection and collected with heparinized hematocrit tubes. Plasma was obtained following centrifugation at 3,000 rpm for 5 min. Plasma was removed and transferred into aprotinin-coated vials and stored at -70°C until VTG analyses. Due to the low amount of plasma collected per fish, 8 fish in each replicate were pooled (2 samples per treatment group). VTG levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit purchased from Biosense Laboratories (Bergen, Norway). The monoclonal antibody used for the assay was made specifically against carp VTG. The positive control was a single intraperitoneal injection of 5 µg/g of 17 α-ethynylestradiol (Sigma Chemical) (Denslow et al. 1999).

All endpoints were tested for statistical significance using a single factor one-way analysis of variance (ANOVA) followed by Tukey-Kramer HSD or Least Significant Difference to identify treatment groups that were significantly different ($p<0.05$). Homogeneity of variances and normality among replicates were determined by Bartlett's test and Komogorov-Smirnov test, respectively.

RESULTS AND DISCUSSION

There were no major differences in water quality between the wastewaters except for conductivity and hardness (Table 1). Conductivity and hardness were elevated in all treatments compared to the control. During the fathead minnow and *Ceriodaphnia* 7-d studies, the control hardness was adjusted to approximate the treatment groups. All other parameters were within acceptable physiological limits.

The results of the 7-d fathead minnow embryo/larvae and *Ceriodaphnia* bioassays are presented in Table 2. The ST and PM samples were highly toxic to fathead minnow embryo/larvae. Conversely, these samples did not affect *Ceriodaphnia* survival or reproduction. No significant toxicity to fathead minnow embryo/larvae was observed following exposure to 10% LL, however *Ceriodaphnia* reproduction was significantly decreased compared to controls.

The ecological relevance of this decrease remains uncertain because the reproduction was close to the EPA acceptability criteria of 15 or more neonates

Table 1. Chemical and physical characteristics during aquatic toxicity bioassays with wastewaters.

	Temperature (°C)	D.O. (mg/L)	Conductivity (µmhos/cm)	Hardness (mg/L)[a]	pH
Ceriodaphnia/fathead minnow[b]					
Control					
Mean[c]	23.1	5.5	403	112	7.86
(SEM)	(0.1)	(0.3)	(14)	(1)	(0.12)
10%LL					
Mean[c]	23.3	5.1	434	55	8.02
(SEM)	(0.1)	(0.4)	(8)	(1)	(0.06)
ST					
Mean[c]	23.3	4.4	338	67	7.74
(SEM)	(0.1)	(0.3)	(24)	(1)	(0.07)
PM					
Mean[c]	23.0	4.7	373	104	7.87
(SEM)	(0.1)	(0.6)	(78)	(26)	(0.15)
Common Carp					
Control					
Mean[c]	23.7	5.7	176	25	6.81
(SEM)	(0.2)	(0.1)	(4)	(2)	(0.05)
10%LL					
Mean[c]	23.4	5.5	405	78	7.66
(SEM)	(0.1)	(0.1)	(14)	(24)	(0.04)
ST					
Mean[c]	23.3	5.5	397	96	7.47
(SEM)	(0.1)	(0.1)	(10)	(11)	(0.03)
PM					
Mean[c]	23.2	5.5	437	119	7.48
(SEM)	(0.1)	(0.1)	(11)	(28)	(0.04)

[a]Total hardness as $CaCO_3$ (dark coloration in the PM samples may have resulted in high variability from the colorimetric method). [b]No differences between bioassays were observed and values were combined. [c]Mean values (± standard error of the mean).

per surviving adult. Regardless, fathead minnow embryo/larvae were more sensitive to the undiluted ST and PM samples. Both of these wastewaters are complex mixtures that are known to contain large amounts of organic and inorganic compounds. The toxicity of the ST effluent enters a major river and, in theory, is diluted to levels where no acute toxicity occurs. However, the PM samples were taken from a creek 3.5 km from the discharge point. Apparently, the dilution in the creek at the point of sample collection was not sufficient to ameliorate the toxicity to fathead minnow embryo/larvae.

Table 2. Effects of effluents on 7-d fathead minnow (*Pimephales promelas*) embryo/larvae and *Ceriodaphnia dubia* survival and reproduction.

	Fathead minnow embryo/larvae[a]		*Ceriodaphnia dubia*	
	% Mortality	% Deformities	% Mortality	Reproduction[b]
Control	17	4	0	26 (1)
10% LL	7	18	0	14 (1)[c]
ST	97[c]	100[c]	0	20 (3)
PM	100[c]	27[c]	10	22 (3)

[a]Larvae deformities consisted of skeletal abnormalities, i.e., bent spine. [b]Total number of neonates per surviving adult (mean; standard error of the mean). [c]Statistically significant from *Control* at $p<0.05$.

Untreated landfill leachates also have the potential to be highly toxic to organisms if they leach into aquatic ecosystems. Landfill leachates containing untreated industrial and municipal wastes collected within the city limits of Seoul, Korea, were highly toxic in the Japanese Medaka (*Oryzias latipes*) embryo-larval assay (Kaur et al. 1996). The Medaka LC_{50}s obtained from samples collected from several sites along this landfill ranged from 1.6 to 2.4% (v/v). Although an LC_{50} study was not conducted with fathead minnow embryos and larvae, the LL LC_{50} is greater than 10%.

The results from the 42-d carp study are presented in Table 3. There was a relatively equal distribution of male and female juvenile carp between exposure groups and no sex differences were observed between the endpoints. There were no significant differences in body wt, standard length, LSI, GSI or hematocrit values between wastewater and control groups. However, there was a decrease in condition factor in the PM group (significant at $p<0.1$) and significant decreases in SSI in carp exposed to the LL and PM samples ($p<0.05$). No significant differences were observed in plasma VTG levels (results not presented in table; positive control, negative control, LL, ST, and PM values were 53.20, 1.33, 0.98, 0.015, and 0.523 mg/mL, respectively). Although the VTG screening results represent an average of 2 pooled replicate samples per exposure due to the low amount of plasma collected per fish, they indicate that wastewaters used in this study were not estrogenic to common carp juveniles.

There is considerable variation in the literature concerning the endocrine effects in aquatic organisms following exposure to industrial and domestic waste streams. Many of these apparent discrepancies between studies involve differences in exposure designs, species, and/or wastewater treatment technology and discharge volumes. As mentioned, none of the exposure groups in the present study indicated an estrogenic effect to juvenile carp based on preliminary VTG levels. Similar results have been reported in fathead minnows and goldfish exposed to municipal wastewater treatment effluent discharges (Nichols et al. 1999; Giesy et al. 2003) or indigenous mosquitofish below a wastewater treatment plant (Angus

Table 3. Effect of effluents on condition factor, relative organ weights, and hematocrit of juvenile common carp (*Cyprinus carpio*) exposed for 42 days.

	Body Weight (g)	Standard Length (mm)	Condition factor[a]	LSI[b]	SSI[c]	GSI[d]	Hematocrit (%)
Control							
Mean[e]	10.76	74	2.52	1.30	0.200	2.73	42
(SEM)	(1.32)	(3)	(0.03)	(0.04)	(0.013)	(0.73)	(1)
Landfill							
Mean[e]	11.02	75	2.53	1.30	0.166[f]	1.46	42
(SEM)	(0.84)	(2)	(0.05)	(0.04)	(0.007)	(0.29)	(1)
Sewage							
Mean[e]	11.62	75	2.57	1.36	0.179	1.56	39
(SEM)	(1.41)	(3)	(0.04)	(0.05)	(0.011)	(0.25)	(1)
Creek							
Mean[e]	11.90	78	2.40[g]	1.29	0.160[f]	2.38	41
(SEM)	(1.09)	(2)	(0.04)	(0.08)	(0.006)	(0.71)	(1)

[a]Relative condition factor (total body wt x 10^5/standard length^3). [b]Liver-Somatic Index (liver wt x 100/total body wt). [c]Spleen-Somatic Index (spleen wt x 100/total body wt). [d]Gonad-Somatic Index (gonad wt x 100/total body wt). [e]Mean values (± standard error of the mean; n = 16). [f]Statistically significant from Control at $p<0.05$. [g]Statistically significant from control at $p<0.1$.

et al. 2002). However, other investigators have measured VTG induction in carp collected near sewage treatment discharges (Solé et al. 2000), as well as decreases in secondary sex characteristics and GSI in fathead minnows (Hemming et al. 2001). In addition, endocrine disruption has also been observed in fish following exposure to pulp and paper mill effluents (Parrott et al. 2004; Orlando et al. 2002). Although there are regional hotspots such as pesticide contamination in Lake Apopka, Florida (Toft and Guillette 2005), the antiandrogenic and/or estrogenic action of industrial and domestic waste streams does not appear to be a common phenomenon. VTG and sex steroid levels in adult male carp (*Cyprinus carpio*) collected at 25 sites similar to those in the present study did not indicate significant endocrine modulation (Goodbred et al. 1996).

Few studies are available that evaluate the immunotoxicity of wastewater samples. In the present study, the most sensitive biological indicator in carp following exposure to the 10% LL and PM samples was a significant decrease in SSI. These results indicate a potential immunosuppressive action of these wastewaters. Fatima et al. (2001) exposed *Channa punctatus* (Bloch) to 1% concentration of pulp and paper mill effluent for up to 90 d. They observed a significant decrease in the wt of lymphoid organs (i.e., spleen, pronephros, and total kidney), as well as a decrease spleen and pronephros cellularity. Another indicator of immunotoxicity, the plaque-forming cell response, was reduced in the spleen and pronephros after 30 d exposure. These results show immunosuppression of chemical constituents, such as halogenated aromatic hydrocarbons (HAHs) and heavy metals, in the pulp and paper mill effluent.

The samples selected in this study were from three different waste treatments with variable dilutions. The ST sample was undiluted, the LL sample was diluted due to high acute toxicity, and the surface water sample collected downstream from a pulp and paper mill treatment lagoon had an unknown dilution. It is virtually impossible to determine the potential toxicity of these complex mixtures based exclusively on physical and chemical characteristics. However, the use of traditional NPDES bioassays appear sensitive enough to determine the short-term toxicity of these samples (accounting for the acute toxicity of the LL sample to *Ceriodaphnia* neonates). None of the samples were estrogenic to juvenile carp but the LL and PM samples may have exhibited an immunomodulatory effect. Future studies will involve the incorporation of additional immunological endpoints, such as lymphoid cellularity and antibody response assays, at higher dilutions to assess the sensitivity of these immunological endpoints to wastewater exposure. These endpoints may be useful in identifying subtle chronic sublethal effects of wastewater discharges to aquatic ecosystems.

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