Arch Environ Contam Toxicol (2008) 54:31–35
DOI 10.1007/s00244-007-9059-4

Assessing Caffeine as an Emerging Environmental Concern Using Conventional Approaches

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Published online: 24 October 2007
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Abstract Organic wastewater contaminants, including pharmaceuticals, caffeine, and nicotine, have received increased scrutiny because of their detection in water bodies receiving wastewater discharge. Despite recent measurement in United States streams, caffeine's effect on freshwater organisms is not well documented. The present study measured caffeine's lethal and sublethal effects on the freshwater species, *Ceriodaphnia dubia, Pimephales promelas*, and *Chironomus dilutus.* These organisms, which are used in standard testing or effluent monitoring, were exposed to aqueous caffeine solutions under static exposure for 48 hours and daily renewed static exposure for 7 days. Averaged responses of 48-hour acute end points indicated that *C. dubia* was more sensitive to caffeine exposures (LC_{50} = 60 mg/L) than either *P. promelas* (LC_{50} = 100 mg/L) or *C. dilutus* (LC_{50} = 1,230 mg/L). Exposure-response slopes confirmed these findings (3% mortality/mg/L for *C. dubia*; 0.5% mortality/mg/L for *P. promelas*; and 0.07% mortality/mg/L for *C. dilutus*). Comparative 7-day responses between *C. dubia* and *P. promelas* (LC_{50} = 46 and 55 mg/L, respectively) were more similar than the broad range of acute values. Sublethal effects measured for caffeine exposure included impaired *C. dubia* reproduction (IC_{50} = 44 mg/L) and inhibited *P. promelas* growth (IC_{50} = 71 mg/L). According to the results of this study, combined with earlier studies reporting environmental concentrations and product half-lives, caffeine should pose negligible risk for most aquatic vertebrate and invertebrate organisms.

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Organic wastewater contaminants (OWCs) include a broad range of materials, such as pharmaceuticals, pesticides, plasticizers, and various other compounds (Fraker & Smith 2004). Because of the monitored effects of pesticides on nontarget organisms in aquatic receiving systems, pharmaceutical compounds' presence in rivers and streams has produced increased scrutiny on their potential ecotoxicologic effects (Maul et al. 2006). Several studies have reported the presence of caffeine in surface and ground waters across the world (Weigel et al. 2002; Buerge et al. 2003; Metcalfe et al. 2003; Weigel et al. 2004; Sankararamakrishnan & Guo 2005; Thomas & Foster 2005). A study by Kolpin et al. (2002) of 139 stream sites in the United States evaluated the occurrence of 95 different OWCs. Of those contaminants, caffeine was the fourth most frequently detected, occurring in as many as 70% of collected samples, although most were <1 μg/L in concentration. Such prevalence was recently recognized by Metcalfe et al. (2003) in their use of caffeine as a marker compound for human excrement in determining the presence of prescription and nonprescription drugs in surface-water bodies.

Caffeine (3,7-dihydro-1,3,7-trimethyl-1h-purine-2,6-dione) has been coined the most commonly consumed

stimulant by humans (Lawrence et al. 2005). Medicinally it is used as a cardiac, cerebral, and respiratory stimulant, and it also functions as a diuretic (Buerge et al. 2003). More common uses of caffeine, and likely significant sources of environmental contamination, include it being a key ingredient in coffee, tea, chocolate, and soft drinks. Buerge et al. (2003) reported an estimated global average consumption of 70 mg caffeine/person/d. Based on global population and average consumption estimates, 460,000 kg caffeine are consumed daily by humans. In the United States alone, the daily average per-person consumption rate of 210 mg (Buerge et al. 2003) approaches a national total of 63,000 kg caffeine/d. An unknown portion of this consumption makes its way through wastewater-treatment plants globally, and some caffeine will inevitably be deposited into aquatic receiving systems. Although these are sufficient reasons to examine the toxicologic effects of caffeine on nontarget organisms, this study actually originated from agricultural research examining novel seed treatments for rice. Avery et al.'s (2005) determination of caffeine as an effective and economic rice seed treatment to deter blackbirds prompted additional toxicity tests to confirm caffeine's use as a seed treatment.

The current need for additional information on caffeine toxicity has accompanied increasing concerns about its prevalence in and mixture with other wastewater components. Bantle et al. (1994) found that caffeine concentrations in water were high enough to affect *Xenopus laevis* egg development when exposed for 96 hours (LC_{50} = 0.22 to 0.37 mg/mL). The International Uniform Chemical Information Database (2004) reported 120-hour *X. laevis* LC_{50} values between 0.13 and 0.19 mg/mL. Further research is needed to ensure that caffeine levels present in contaminated streams do not present a risk to freshwater species. The objectives of this study were to compare, contrast, and model responses of populations of *Ceriodaphnia dubia* (water flea), *Chironomus dilutus* (midge), and *Pimephales promelas* (fathead minnow) to 48-hour exposures of caffeine. *C. dubia* and *P. promelas* were also exposed to caffeine for 7 days to assess the effects of caffeine on survival, reproduction (*C. dubia*), and growth (*P. promelas*).

Materials and Methods

Caffeine stock solution was prepared by dissolving known quantities of 100% caffeine powder (10 g; Fisher Scientific) into Milli-Q water (1 L) (Table 1). Because of caffeine's photosensitivity, stock solutions were covered in aluminum foil when not in use, and fresh solutions were made every other day throughout test duration. To achieve target caffeine concentrations ranging from 0.01 to 1.6 g/mL, serial dilutions were performed using caffeine stock solution and moderately hard water (formulated at the Arkansas State University Ecotoxicology Research Facility [ASUERF]). All caffeine concentrations reported in the current study were nominal and were not confirmed analytically.

Test procedures and conditions were conducted according to static acute and static-renewal chronic methods outlined by United States Environmental Protection Agency (USEPA) (2002a, 2002b). All organisms were cultured on site at ASUERF. Toxicity experiments (repeated three times for each species) were conducted at 25 °C ± 1°C under a 16:8 light-to-dark photoperiod. Aqueous physicochemical parameters (pH, temperature, dissolved oxygen, conductivity, alkalinity, and hardness) were measured when water was first prepared and introduced to test containers and after it was removed for renewal from the previous day. All toxicity assay results were analyzed statistically using Toxcalc (version 5.0.25; McKineyville, CA).

C. dubia

C. dubia (<24 hours old) were exposed to a control solution (moderately hard water only) and five caffeine concentrations (0.04, 0.05, 0.06, 0.07, and 0.08 g/mL) for 48-hour survival and 7-day survival and reproduction assessments. For 48-hour assessments, five *C. dubia* were placed in each of four replicate 50-mL containers (filled with 30 mL control or caffeine-amended water; 20 total organisms exposed/concentration). Survival and reproduction assessments (7 days) used one *C. dubia* placed in each of 10 30-mL replicate containers for each treatment (15 mL aqueous volume added). For 7-day assessments, water was renewed daily from prepared dilutions, and survival and neonates were counted daily. Organisms were then transferred by pipette into the renewed water containing food. *C. dubia* were fed a 200-µL suspension of *Selenastrum capricornutum* and *Chlorella vulgaris* and 100 µL YCT (yeast, trout chow, and cerophyll)/test chamber daily. Temperatures of old and new water were within 1°C. After 7 days, neonates produced in each replicate were totaled

Table 1 Chemical characteristics of caffeine

Molecular weight: 194.19[a]
Water solubility: 1 g / 46 mL[b]
Specific gravity: 1.23[b]
Boiling point: 178°C[b]
Melting point: 238°C[b]
Log K_{ow}: 0.01[c]

[a] ChemFinder 2004

[b] Baker 2006

[c] Gossett et al. 2003

and compared with controls to determine if caffeine affected reproduction. End-point values were obtained using a hypothesis test approach with Fisher's exact test, Dunnett's procedure, or Steel's many-one rank Test (USEPA 2002b). Tests for normality and homogeneity of variance included Shapiro-Wilk's and Bartlett's tests, respectively.

P. promelas

P. promelas (<24 hours old) were exposed to five different caffeine concentrations (0.01, 0.02, 0.05, 0.1, and 0.2 g/mL); one control, containing moderately hard water only, was made on site. For both 48-hour and 7-day toxicity assessments, 4 replicates with 10 fish each were placed in 250-mL acid-washed test containers with glass dishes as covers (80 x 100 mm; Corning 3250). Approximately 200 mL water was added in each container at the start of the test, and water renewal (7-day assessments only) was performed by siphoning half of the water from the container and replacing with the same amount of newly prepared water, with care taken not to disturb exposed organisms. This deviates slightly from USEPA-recommended procedures of 250 mL overlying water for chronic assessments and replacement of 80% to 85% water daily. Deviations are noted as part of the ASUERF standard operating procedures. Changes in water volume replacement were made to insure minimal organism injury and stress. During 7-day assessments, test organisms were fed three times daily by introducing approximately 1 drop *Artemia* (brine shrimp) to each container. Survival was recorded daily, and after 7 days, the remaining fish were transferred into aluminum pans, killed, and dried for weighing. Statistical assessments used were similar to those for *C. dubia.*

C. dilutus

To prevent possible binding of caffeine to soil particles, glass beads were used instead of reference sediment to serve as a substrate for burrowing. This ensured that the aqueous caffeine mixture was the primary medium interacting with *C. dilutus*. Glass beads (150 to 212 μm, 4 to 5 g dry weight; Sigma Chemical, St. Louis, MO) were soaked in 25 mL Milli-Q water for at least 12 hours before test setup and initiation. A volume of 150 mL of the five prepared concentrations (0.7, 0.9, 1.1, 1.3, and 1.6 g/mL) and control water were each added to 250-mL wide-mouth borosilicate glass beakers. Ten third-instar larval midges (13to 15 days old) were added by pipette to the test container. To limit the use of stressed organisms, *C. dilutus* were left in their casing, and any floating midges were replaced at test initiation. Survival was recorded after 48 hors. Because of the short duration of these tests, organisms were not fed to ensure limited interaction and association of caffeine with other particles. Normality assumptions were tested using Shapiro-Wilk's test and Steel's many-one rank test to compare variation in survival among sites (alpha = 0.05).

Results and Discussion

In the current study, *C. dubia* was the most sensitive test species to caffeine, with a mean 48-hour LC_{50} (±SE) of 57 ± 3.3 mg/L. *P. promelas* and *C. dilutus* followed in sensitivity, with mean LC_{50} values of 97 ± 12 mg/L and 1233 ± 159 mg/L, respectively (Table 2). Exposure–response relations were also determined according to methods used by Moore et al. (1998), where slopes illustrate the response (mortality) elicited per unit of concentration in excess of the lower threshold (20% mortality) (Fig. 1). For 48-hour assessments, *C. dubia* had a slope of 3% mortality/mg/L, followed in sensitivity by *P. promelas* (0.5% mortality/mg/L) and *C. dilutus* (0.07% mortality/mg/L). Seven-day exposures of both *C. dubia* and *P. promelas* to caffeine resulted in slightly increased lethal toxicity compared with 48-hour exposures, with mean 7-day LC_{50} values of 47 ± 3 mg/L and 57 ± 3 mg/L, respectively, for the two organisms (Table 2). Exposure–response slopes for 7-day assessments of *C. dubia* and *P. promelas* were 4% mortality/mg/L and 1% mortality/mg/L, respectively (Fig. 2). Sublethal biologic measurements (*e.g.*, inhibition concentrations [IC_{25}]) were calculated for 7-day exposures, with *C. dubia* responding at 40 ± 0 mg/L, whereas *P. promelas* first indicated inhibition

Table 2 Toxicity end points for test species exposed (during three separate experiments) to aqueous concentrations of caffeine[a]

Organism	48-hour LC_{50} (mg/L)	7-day LC_{50} (mg/L)	7-day EC_{25} (mg/L)
C. dubia	50 (40–50)	50 (50–60)	40 (20–50)
C. dubia	60 (50–60)	50 (40–50)	40 (20–40)
C. dubia	60 (50–70)	40 (40–50)	40 (40–40)
P. promelas	120 (100–130)	60 (50–80)	30 (30–40)
P. promelas	80 (10–2720)	50 (0–80)	30 (20–40)
P. promelas	90 (70–110)	60 (60–70)	90 (0–90)
C. dilutus	1,520 (850–10610)	NA	NA
C. dilutus	1,210 (1010–1640)	NA	NA
C. dilutus	970 (510–1160)	NA	NA

NA = not applicable (indicates that particular tests were not conducted with *C. dilutus*)

[a] 95% confidence intervals are included in parentheses

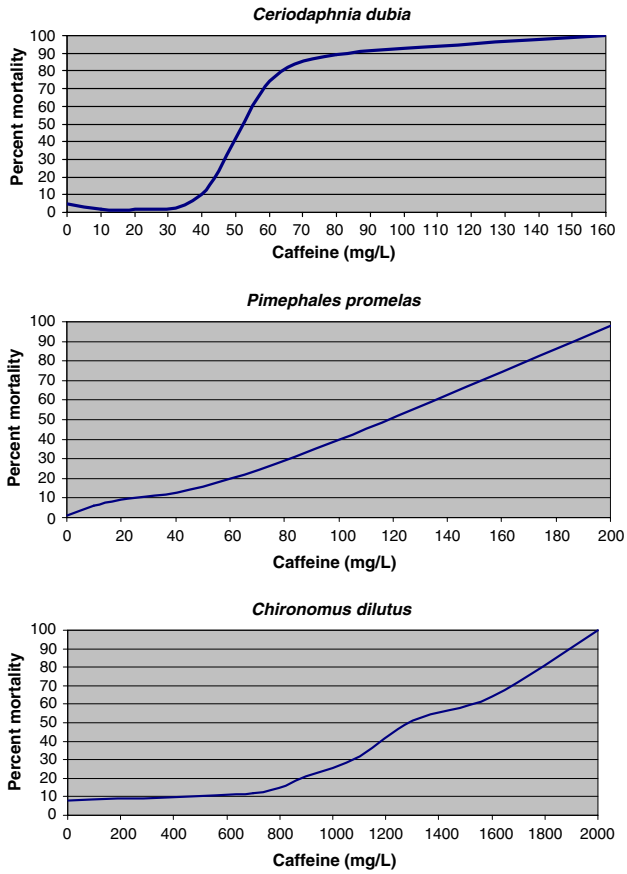

Fig. 1 Forty-eight-hour exposure-response slopes for *C. dubia, P. promelas*, and *C. dilutus* exposed to caffeine

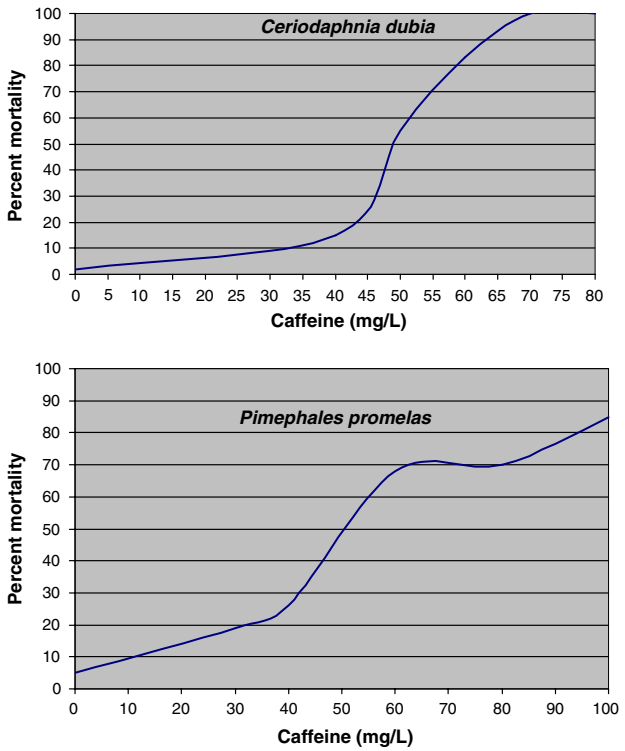

Fig. 2 Seven-day exposure-response slopes for *C. dubia* and *P. promelas* exposed to caffeine

at 50 ± 20 mg/L. One-sample Student *t* test was used to compare 7-day IC_{25} values between vertebrates and invertebrates. Significant differences were reached only at $\alpha = 0.1$, but not at the conventionally used $\alpha = 0.05$, with $p = 0.088$.

Caffeine concentrations resulting in lethal and sublethal effects on aquatic organisms were greater than those detected in surface waters globally. Since 2000, several studies have measured caffeine's presence in surface waters worldwide. Kolpin et al. (2002) sampled 139 stream sites in the United States and reported a median detection caffeine concentration of 0.1 µg/L. Metcalfe et al. (2003) used caffeine as a marker for human excrement and reported the highest mean caffeine concentration from five Ontario (Canada) sewage-treatment plant (STP) effluents to be 0.677 µg/L. Studies by Thomas and Foster (2005) and Batt et al. (2006) examined STP effluents in Virginia and New York, respectively. According to Thomas and Foster (2005), caffeine STP effluent concentrations ranged from 0.013 to 0.036 µg/L. Reported caffeine concentrations from New York STP effluents ranged from 0.19 to 9.9 µg/L (Batt et al. 2006). Buerge et al. (2003) reported caffeine concentrations in Swiss lakes and rivers from 6 to 250 ng/L, whereas Swiss STP effluents ranged from 0.03 to 9.5 µg/L caffeine.

Kolpin et al. (2004) examined 76 water samples upstream and downstream from certain towns and cities in Iowa and examined OWC concentrations during high-, normal-, and low-flow conditions. Caffeine was detected in 83% of high-flow samples (maximum concentration of 0.078 µg/L), 83% of normal-flow samples (maximum concentration of 0.036 µg/L), and 57% of low-flow samples (maximum concentration of 1.39 µg/L). Sankararamakrishnan and Guo (2005) compared caffeine concentrations from Deal Lake, NJ, during wet and dry weather patterns. Dry-weather caffeine concentrations ranged from 0.16 to 0.27 µg/L, whereas wet-weather concentrations increased from 0.25 to 45 µg/L. In addition, these investigators ran a Pearson's correlation with caffeine against several other indicators of human excrement. A correlation of 1.0 was derived for caffeine: fecal coliforms, whereas correlations of 0.97 were derived for caffeine: enterococci and caffeine: fecal streptococcus (Sankararamakrishnan & Guo 2005).

The majority of available ecotoxicologic data on caffeine is associated with embryonic development of *X. laevis* (African clawed frog). Extensive work conducted with this species in the mid-1990s resulted in 4-day caffeine EC_{50} values (concentration resulting in malformation of 50% of embryos) ranging from 0.074 to 0.158 mg/mL (Bantle et al. 1994). This same study reported 4-day

caffeine LC_{50} values ranging from 0.24 to 0.35 mg/mL. De Young et al. (1996) compared the effects of 5-day caffeine exposures on the embryonic development of both *X. laevis* and *P. promelas*. Although the *P. promelas* 5-day LC_{50} was nearly four times greater than that of *X. laevis* (720 mg/L vs. 190 mg/L), other sublethal end points were more sensitive for *P. promelas*. Mean 5-day EC_{50} and LOEC values for *P. promelas* were 70 and 20 mg/L, respectively, compared with those for *X. laevis*, which were 130 and 80 mg/L, respectively.

Based on these results, caffeine does not seem to be a threat for freshwater organisms given its current presence in the aquatic environment. Although it may be possible that caffeine levels in streams may persist such that they will have potential for long-term exposure effects, this is unlikely given that its mean half-life is approximately 1.5 days (Lam et al. 2004). However, Thomas and Foster (2005) argued that even a quickly degradable drug can act as a persistent chemical. If caffeine is profusely discharged from anthropogenic sources into an environment, it could constantly replenish levels regardless of the amount of caffeine degraded, creating a dynamic equilibrium. With this possibility in mind, future ecotoxicologic research might include potential synergistic, additive, or antagonistic effects of caffeine with a host of other commonly detected OWCs.

Acknowledgments All programs of the United States Department of Agriculture (USDA) are offered on a nondiscriminatory basis without regard to race, color, national origin, religion, sex, marital status, or handicap. Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

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