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Microbial Reduction of 9-Fluorenone to 9-Hydroxyfluorene in Carbon-Filtered Water: A Confounding Factor in an Aquatic Bioassay of 9-Fluorenone with Larvae of the Midge, *Chironomus tentans* (Fabr.)

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When investigations are carried out on the aquatic toxicity of an organic pollutant, the concentration of the pollutant in water is not always determined after completion of an exposure experiment. However, in the case of chemicals that can be degraded by either abiotic or biotic processes, the absence of concentration data can lead to erroneous conclusions about the toxicity of the pollutant (Rufli et al. 1998). In our laboratory, several oxidized polycyclic aromatic hydrocarbons (oxy PAHs) have been identified in sediment samples collected from the Massena, NY area of the St. Lawrence River near an outfall from an aluminum smelter. When one of these compounds, 9-fluorenone, was tested for toxicity to larvae of the midge *Chironomus tentans* it was found that there was a greater than 90% reduction of 9-fluorenone to 9-hydroxyfluorene (Figure 1). In the study reported here, the experimental parameters responsible for the reduction were determined.

MATERIALS AND METHODS

Aliquots from dimethyl sulfoxide (DMSO) solutions of 9-fluorenone (Sigma-Aldrich, Milwaukee, WI) were spiked into 200 ml of water in 250-ml aluminum foil-covered beakers. Spiking levels in initial experiments were 2mg/L but were later reduced to levels between 30 and 80 μg/L. The solutions were maintained at room temperature for varying periods of time. In experiments where food was used, the food source was 25 mg/beaker of either TetraFin (TetraWerke, D 49304 Melle, Germany) or freeze-dried salmon from the US Pacific Coast. Reconstituted water was prepared from Milli-Q UV Plus purified water (Millipore Corporation, Bedford, MA, USA) and salts, as recommended by the USEPA (USEPA 2000). Culturing of *Chironomus tentans* larvae (referred to as chironomids) was carried out under static conditions in 42 L aquaria using USEPA protocols (USEPA 2000). Municipal water (referred to as tap water) for use in culturing and in bioassay experiments was filtered though granulated activated carbon (Petcetera Inc., Bayonne, NJ). Microorganisms were filtered from water samples under completely aseptic conditions.

The contents of each beaker were extracted with three 50-60 ml portions of methylene chloride and were solvent-exchanged to xylene (1 ml). Samples spiked at the 30-80 μg/L level with 9-fluorenone were spiked with a similar quantity of the internal

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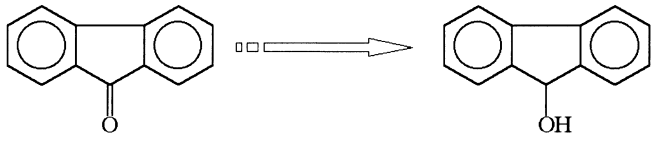

A B

Figure 1. Schematic for the reduction of 9-fluorenone (A) to 9-hydroxyfluorenol (B).

standard, D_8-fluorenone (prepared from the oxidation of D_{10}-fluorene according to the procedure of Chidambaram and Chandrasekaran (1987)), before extraction. Sample extracts were analyzed with a Hewlett-Packard 5970 Mass Selective Detector connected to a J&W DB-XLB fused-silica capillary column with dimensions of either 60 m x 0.25 mm I.D. or 30 m x 0.25 mm I.D. Equal quantities of the alcohol and the ketone were baseline-separated on the 60-m column, whereas there was a 20% overlap on the 30-m column. Major ions (m/z) and their % of the base peak in the mass spectra of 9-fluorenone and 9-hydroxyfluorene standards were as follows: for 9-fluorenone 180 (100) $(M)^+$, 152 (40) $(M\text{-}CO)^+$; for 9-hydroxyfluorene 182 (55) $(M)^+$, 181 (75) $(M\text{-}H)^+$, 152 (45) $(M\text{-}CH\text{-}OH)^+$. Samples spiked with 2 mg/L 9-fluorenone were analyzed by scanning from m/z 50 to m/z 500 after 10X dilution, and samples spiked with 30-80 µg/L were analyzed by selected ion monitoring of m/z 180, 181, 182 and 188 (D_8-fluorenone). Concentrations of 9-hydroxyfluorene were calculated from the relative responses at m/z 181 and m/z 188, and concentrations of 9-fluorenone were calculated from the relative responses at m/z 180 and m/z 188, using appropriate relative response factors between the native compounds and the deuterated standard. The use of D_8-fluorenone as a standard for both compounds is based on the premise that the recoveries of 9-fluorenone and 9-hydroxyfluorene from aqueous solutions are similar. The recoveries of 9-hydroxyfluorene and 9-fluorenone were approximately 70%.

RESULTS AND DISCUSSION

Initial experiments were carried out to determine whether the water source for the bioassay, the food, or the chironomids were involved in the reduction process (Table 1). When water was obtained from a carbon filter that had been in use for only 24 h, there was minimal reduction of 9-fluorenone (6%) over a 72-h period, and the reduction was increased only slightly in the presence of food. However, when water was obtained from a filter that had been in use for 1 yr, 42% of the 9-fluorenone was reduced to 9-hydroxyflourene, and this was increased to 83% in the presence of food and chironomids. These results suggested that bacterial growth occurred in the filter over the 1-yr time period during which the filter had been in use, and that one or more of the bacterial species were involved in the reduction reaction. While charcoal filtration is a technique that can be applied to the removal of chlorine from municipal water supplies used in aquatic bioassay experiments (USEPA 2000), bacterial growth

Table 1. The influence of the water source and the presence/absence of food (boiled freeze-dried salmon) and chironomid larvae on the reduction of 9-fluorenone to 9-hydroxyfluorene.

Exposure conditions	Reduction (%)		Mortality (%)	
	48 h	72 h	48 h	72 h
Charcoal-filtered tap water (24 hr filter use)		6		
Charcoal-filtered tap water (24 hr filter use) + food		10		
Charcoal-filtered tap water (1 yr filter use)		42		
[a] Charcoal-filtered tap water (1 yr filter use) + food + chironomids		83	nd	50
Reconstituted water		1		
Reconstituted water +food	12	46		
Reconstituted water +food + chironomids	77	87	11	67

[a] This exposure was carried out with 2 mg/L 9-fluorenone. All other exposures were carried out with 9-fluorenone concentrations in the range of 30 - 80 µg/L. nd = not determined.

has been documented to occur in granulated activated carbon used for water treatment in municipalities (Camper et al. 1986), hospitals (Morin 2000), pharmaceutical manufacturing (Kawai et al. 2004) and in point-of-use (POU) filters in the home (Chaidez and Gerba 2004).

In our experiments, bacterial growth appears to have been enhanced by the presence of food and chironomids. While the chironomids were washed four times with distilled water after removal from the culture tank, before use in the experiments, the culture-tank water was prepared from charcoal-filtered tap water that was renewed on a weekly basis. During the renewal process, a minimal quantity of water was left in the tank before the addition of fresh water. It is apparent from the results shown in Figure 2 that this incomplete water exchange resulted in the growth of bacteria that were capable of reducing 9-fluorenone at a faster rate than the water collected directly from the carbon filter. Therefore, despite the washing process, some of the bacteria from the culture tank water could have been adhering to the larvae. This could explain the enhanced reduction in the presence of chironomids, although reduction by the chironomids themselves cannot be ruled out. The mortality data shown in Table 1 should only be considered as indicative of the acute toxicity of 9-fluorenone/9-hydroxyfluorenone to *Chironomus tentans*, as there were only nine larvae per test, and there were no control larvae.

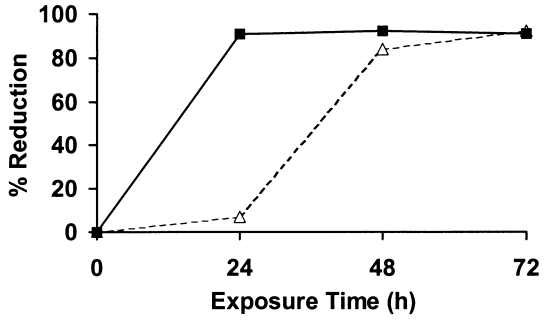

Figure 2. Reduction of 9-fluorenone (30 µg/L) to 9-hydroxyfluorenol in a chironomid larval culture tank (■) and in charcoal-filtered tap water + freeze-dried salmon (△).

Reconstituted water, prepared using high-purity distilled-deionized water and inorganic salts, is an alternate water source for bioassay experiments. We used deionized water that had been subjected to carbon and resin filtration followed by high-energy UV radiation for preparation of reconstituted water. While this high-purity water is not considered to be sterile, the manufacturer of the purification unit indicates that "at 254 nm the powerful UV lamp also kills bacteria, ensuring minimal bacterial growth on wet surfaces and in purification media" (Technical Brief , Millipore Corporation). After the appropriate salts had been added, solutions of 9-fluorenone were maintained in the reconstituted water for periods of 48 and 72 h, in the presence and in the absence of food (Table 1). Only a trace amount of 9-hydroxyfluorene was found in the reconstituted water solution after 72 h. However, in the presence of food, the extent of reduction was increased to 12% and 46%, after 48 and 72 h, respectively. The preparation of the reconstituted water was not carried out under aseptic conditions, and it is possible that contamination with a small number of bacteria occurred during this process, and that these bacteria then proliferated in the presence of food.

Additional evidence for the role of bacteria in the reduction reaction was obtained by heating the food media and the charcoal-filtered tap water. Heating of the food (freeze-dried salmon) had only a minimal effect on the reduction, but no measurable reduction occurred when the charcoal-filtered water was heated (Table 2). Included in Table 2 are data from a 72-h experiment with a solution of 9-fluorenone in charcoal- filtered tap water containing TetraFin, the commercial fish food generally used in our laboratory for bioassays with chironomids. The reduction reaction was enhanced to the same extent with this food source as with the freeze-dried salmon.

Table 2. The influence of the food source and heat (30 min at 100 °C) on the reduction of 9-fluorenone (2 mg/L) to 9-hydroxyfluorene in charcoal-filtered tap water [a].

Exposure conditions	Reduction (%)
Charcoal-filtered tap water + TetraFin fish food	84, 83
Charcoal-filtered tap water + freeze-dried salmon	78
Charcoal-filtered tap water + boiled freeze-dried salmon	57,69
Boiled charcoal-filtered tap water + freeze-dried salmon	≤1

[a] The filter had been in use for 1 yr.

The freeze-dried salmon was used for the experiments described in this report, as it did not contain any of the additives present in TetraFin (ascorbic acid and Ethoxyquin, an antioxidant) that could potentially affect the reduction reaction. It is apparent from the results in Table 2 that these additives were not involved in the reduction reaction.

The majority of the data presented in Table 1 and all of the data in Fig.2 were obtained from solutions of 9-fluorenone in the concentration range 30-80 µg/L, whereas the data in Table 2 were obtained at a 9-fluorenone concentration of 2 mg/L. The results shown in Table 3 indicate that for charcoal-filtered water, the higher concentration level exceeds the capacity of the bacterial population to effectively reduce 2-fluorenone to 9-hydroxyfluorene. While there is an enhancement in the extent of reduction when food is present, the reduction level is higher in the 80 µg/L solutions than in the 2 mg/L solutions.

Table 3. The influence of the substrate concentration on the reduction of 9-fluorenone to 9-hydroxyfluorenone in charcoal-filtered tap water [a].

Exposure conditions	Reduction (%)			
	2 mg/L		80 µg/L	
	48 h	72 h	48h	72h
Charcoal-filtered tap water		≤1,13		42
Charcoal-filtered water + salmon (boiled)	58	69,57	84	92

[a] The filter had been in use for 1 yr.

Filtration experiments with culture-tank water were carried out under aseptic

Table 4. Determination, by filtration experiments, of the size range of the microorganisms in the culture-tank water responsible for the reduction of 9-fluorenone (30 μg/L) to 9-hydroxyfluorene.

Treatment	Reduction (%)	
	+ food[a]	-food
Culture tank water	95	67
Filtrate, 3-μm filter	95	68
Filtrate, 0.45-μm filter	88	82
Filtrate, 0.22-μm filter	3	5
Boiled culture-tank water	2	7

[a] Food was boiled freeze-dried salmon.

conditions, to provide additional data on the microorganisms involved in the reduction process (Table 4). A 3- μm filter removes protozoa from water samples, and the absence of any change in the level of reduction in the filtrate from this filter compared to the unfiltered culture water suggested that protozoa were not involved in the reduction. Most bacteria in water samples are removed by a 0.45-μm filter, and again the absence of any significant change in reduction in the filtrate from this size filter suggested that a small bacterium or mycoplasmid was involved in the reduction process. These small organisms can be removed by filtration through a 0.22-μm filter. Since only a small background level of reduction occurred with the 0.22-μm filtrate, the reduction reaction could have been accomplished by a small bacterium or a mycoplasmid. It has been reported in a recent study by Inoue et al. (1998) that a wild-type strain of the bacterium *Bacillus brevis* reduced 9-fluorenone to 9-hydroxyfluorene in 97% yield at 30°C. However this bacterium should have been removed by the 0.45-μm filter, and additional work will be required to identify the organisms responsible for the reduction in the 0.45-μm filtrate in our study.

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