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Evaluation of *in vitro* Enzyme Inhibition for Screening Petroleum Effluents[1]

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Prediction of environmental hazards increasingly requires simple, rapid, and inexpensive assay methods. This concept has permitted hazard evaluation schemes for both individual compounds and complex effluents (e.g., CAIRNS and DICKSON 1978, CAIRNS et al. 1978, UNITED STATES ENVIRONMENTAL PROTECTION AGENCY 1979). The use of in vitro acetylcholinesterase inhibition to predict ambient organophosphorus concentration was proposed by SARDAR et al. (1970) and GAMSON et al. (1973). Subsequently, RUTHERFORD et al. (1979) proposed using in vitro enzyme inhibition as a simple method to screen petroleum effluents for potential toxicity. Using an artificial refinery mixture (ARM), inhibition of glucose-6-phosphate dehydroenose (G6PDH) was noted within 30 min at an ARM concentration falling between the 24-hr LC50 values for invertebrates and fish.

Later research in our laboratory with the enzymes 6-phosphogluconate dehydrogenose, glutamate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase, mannosidase, α-amylase, acetycholinesterase, and alkaline phosphatase indicated that these enzymes were not suitable for predicting the toxicity of ARM. When effects were observed, they occurred at concentrations 10 to 100 times the ARM formulation. G6PDH, however, consistently gave the best results.

The purpose of this research was two fold: (a) to determine if in vitro inhibition of G6PDH can be used to predict toxicity of actual refinery effluents, and (b) to determine how inhibition of G6PDH varies as the components of an effluent (ARM) vary.

MATERIALS AND METHODS

Daphnia 48-hr and goldfish 96-hr toxicity tests were conducted on a refinery effluent before and after secondary treatment. The toxicity tests were static with no renewal or aeration using the procedures in Standard Methods for the Examination of Water and Wastewater (APHA et al. 1975). Toxicity tests were conducted in duplicate on effluent stored at 6 C within 24 hr of collection.

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The G6PDH enzyme assays on the refinery effluent and the ARM were conducted using the method described by Rutherford et al. (1979). G6PDH (E.C.1.1.1.49) activity was monitored spectrophotometrically (Gilford Model 240) after a 30-min incubation in test solutions.

An arbitrary reference mixture (ARM; Buikema et al. 1976) was prepared at 100 times the original concentration (100 X) using soft water (50 ppm hardness) as diluent. This 100 X ARM was also prepared with one or more of the components deleted for its characterization. Centrifugation, when used, was carried out at 10,000 x g for 10 min after preincubation of the enzyme in the ARM.

RESULTS AND DISCUSSION

Animal bioassays as well as enzyme inhibition tests were performed on a refinery effluent before and after secondary treatment. G6PDH was inhibited about 18% by the untreated effluent. This mixture was quite toxic to both Daphnia and goldfish (Table 1). After secondary

TABLE 1

Enzyme inhibition and LC50 data for Daphnia and goldfish exposed to actual refinery effluents.

Treatment	% Enzyme Inhibition	LC50 (as % of effluent) Hr	Daphnia	Goldfish
Before[a]	18.0 ± 2.9	24	3.57	19.3
		48	0.37	18.3
		96	----[c]	18.3
After[b]	12.4 ± 1.9	24	not toxic	81.0
		48	not toxic	81.0
		96	----	81.0

[a]Chemical parameters: 8.2, pH; 31.7 mg/l, oil and grease; 4.20 mg/l, sulfides; 22.2 mg/l, ammonia; 406 mg/l, BOD; 643 mg/l, COD; 186 mg/l, TSS.

[b]Chemical parameters: 6.97, pH; 7.3 mg/l, oil and grease; 0.72 mg/l, sulfides; 14.2 mg/l, ammonia; 65 mg/l, BOD; 134 mg/l, COD; 67 mg/l, TSS.

[c]96-hr LC50 not calculated.

treatment, inhibition of G6PDH was still detectable, although it was significantly reduced. Daphnia was no longer sensitive to the effluent, and the goldfish 96-hr LC50 was 81% effluent. The enzyme inhibition values obtained after secondary treatment were similar to those obtained for a 1X ARM (RUTHERFORD et al. 1979), which simulated the 1977 guidelines promulgated by the United States Environmental Protection Agency (USEPA 1973).

A much greater enzyme inhibition was expected from the effluent before secondary treatment given the gross chemical characteristics of this effluent (Table 1). From this observation, it was hypothesized that inhibition would vary if effluent components also varied. For example, particulate material (TSS) would be expected to exert an effect depending on its ability to absorb toxic components or the enzyme. We found that removal of TSS from the effluent prior to enzyme incubation resulted in no detectable enzyme inhibition. Further experiments indicated that TSS itself did not bind the enzyme.

We then determined by altering the composition of ARM how effluent components interact to affect G6PDH inhibition. Component concentrations were elevated to 100 X ARM for these tests in order to assess accurately the effect of each component. The inhibitory effect of complete ARM was compared with that of its individual components (Table 2). Sulfide, oil, and kaolinite significantly inhibited G6PDH activity. The three remaining components had no significant effect on the enzyme activity. The effect of the particulate kaolinite was nearly as great as the effect of the total ARM, and its effect could be augmented by centrifugation presumably to remove a G6PDH-kaolinite complex. These results indicate that the G6PDH may be absorbing to the kaolinite, an effect which was not observed with the TSS in the refinery effluent. One reason for this presumed discrepancy is that the TSS of the refinery effluent was principally organic, while the kaolinite is an inorganic aluminum silicate complex.

The ARM was then prepared with individual components deleted. The effect of component deletion on enzyme inhibition was complex, and the results were not totally predictable from the effects observed when the components were tested individually (Table 2). The deletion of oil did not significantly affect inhibition, although oil itself significantly inhibited G6PDH. Phenol had virtually no effect when tested individually or when deleted. Chromium and ammonium, which had no effect individually, significantly contributed to the inhibition of the total ARM. Major effects of sulfide and kaolinite were observed in both the individual and deletion studies although the magnitude of the effects varied (Table 2).

TABLE 2

Effects of ARM, ARM components, and component deletion on G6PDH activity.

Combination	Percent Inhibition	Component	Approx. effect	
			Individual	Combination
Total ARM	86.1 ± 3.2			
ARM minus No. 2 fuel oil	83.7 ± 1.9	Oil	25.4	2.4
No. 2 fuel oil	25.4 ± 0.4			
ARM minus phenol	80.3 ± 2.7	Phenol	1.5	5.8
Phenol	1.5 ± 3.3			
ARM minus ammonium	62.3 ± 1.9	Ammonium	0.0	21.3
Ammonium	0.0 ± 0.0			
ARM minus chromium	36.3 ± 4.7	Chromium	0.5	49.8
Chromium	0.5 ± 1.1			
ARM minus sulfide	33.4 ± 1.6	Sulfide	18.8	50.4
Sulfide	18.8 ± 6.3			
ARM minus kaolinite	27.6 ± 5.2	Kaolinite	75.5	56.1
Kaolinite (not centrifuged)	75.5 ± 1.6			
Kaolinite (centrifuged)	92.4 ± 0.9			

The above results indicate that potential additive, synergistic, and antagonistic interactions occur among ARM components. These interactions were verified by conducting experiments with selected mixtures of ARM components (Table 3). As predicted from deletion studies, when both phenol and oil were removed from the ARM (Mixture A), there was no change in enzyme inhibition.

TABLE 3

Effect of mixtures of ARM components on G6PDH activity.

Combination	Percent Inhibition	Combination	Percent Inhibition
Ammonium, chromium, sulfide, kaolinite, No. 2 fuel oil, phenol (ARM)	86.1 ± 3.2	Chromium, sulfide, kaolinite, No. 2 fuel oil, phenol	62.3 ± 1.9
Ammonium, chromium, sulfide, kaolinite (Mixture A)	89.1 ± 4.0	Chromium, sulfide, kaolinite	89.0 ± 5.6
Ammonium, chromium, sulfide	25.3 ± 5.1	Chromium, sulfide	18.2 ± 5.3
Ammonium, chromium, kaolinite	32.0 ± 2.3	Chromium, kaolinite	40.3 ± 3.0
Ammonium, sulfide, kaolinite	46.2 ± 2.7	Sulfide, kaolinite	74.0 ± 1.9
Ammonium, No. 2 fuel oil	3.7 ± 2.7	No. 2 fuel oil	25.4 ± 0.4

Subsequent deletions of components from Mixture A were made to determine other component interactions. When chromium, sulfide, and kaolinite were individually removed from Mixture A, their percent contributions to inhibition of the enzyme were similar to the results obtained in the original deletion study (Tables 2 and 3). Kaolinite inhibits enzyme activity, but its effect is reduced in the presence of ammonium, chromium, and possibly No. 2 fuel oil. Reduction of inhibition is greatest where ammonium, chromium, and kaolinite are present at the same time. Possibly an ammonium-chromium-alum complex is formed which negates the effect of kaolinite. Sulfide, which is inhibitory alone, appears to act independently of kaolinite, chromium, and ammonium.

When ammonium was removed from Mixture A, it had no effect on enzyme inhibition (Table 3). This was not predicted from the results of the original deletion study (Table 2). It seems that ammonium interactions are complex. For example, ammonium diminishes the inhibition due to No. 2 fuel oil or kaolinite while it increases inhibition due to sulfide (Table 3). Further, ammonium only slightly changes the effect of kaolinite when chromium is present.

From the above discussion it appears that there are three categories of components in ARM. Some are inhibitors, i.e., kaolinite, sulfide, and oil; some modify inhibition, i.e., ammonium and chromium; some are apparently inert, i.e., phenol. Although the interactions of ARM components leading to the inhibition of enzyme activity are complex, these interactions are no different than those observed in effluent acute toxicity test results which fluctuate considerably because of effluent variability. Our results also support the conclusion that protection of aquatic resources cannot be achieved by setting specific water quality criteria.

The application of this method for actual use will require additional development. Our preliminary results indicate that the system is cost-effective and results obtained are comparable to toxicity data (Table 1 and RUTHERFORD et al. 1979). Results were obtained in less than 1 hr at a material cost of 25¢ per test. This system is comparable to the Microtox^{R} system (EMBER 1979) and has utility in screening production processes, waste treatment processes, and effluents. Presently we do not envision that this simplistic system will replace traditional toxicity testing which will be necessary to document environmental hazards.

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