TOXICITY SCREENING OF ENVIRONMENTAL SAMPLES UTILIZING A BACTERIAL BIOASSAY

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ABSTRACT

Preliminary studies have shown that the bioassay based on inhibition of alpha-glucosidase biosynthesis (AGB) in *Bacillus licheniformis* is useful in the acute toxicity screening of environmental samples. Alpha-glucosidase is an extracellular enzyme induced by the presence of maltose in the environment of the cell. The enzyme degrades maltose into glucose residues acting at the 1, 4-alpha-glycoside linkages. Colorimetric measurement of enzyme production is used to determine toxic effects. Earlier studies have shown that the bioassay is comparable with the Microtox assay and appears to be sensitive to the presence of metals in aqueous samples.

Bioassays can be valuable tools in identifying environmental samples that may contain high levels of toxicants such as metals. Using bioassays in preliminary assessments of samples can be a cost-savings step and can help direct future analysis that may involve more expensive chemical tests. We are currently using the *Bacillus licheniformis* AGB bioassay in conjunction with chemical analysis to screen drinking water and aqueous extracts of soil samples collected from three communities in southeast Missouri. Data collected indicated a correlation between elevated metal concentration in the samples and decreased enzyme production in the bioassay.

Key words: *bioassay, acute toxicity, alpha-glucosidase*

INTRODUCTION

The alpha-glucosidase biosynthesis (AGB) assay is a cost-effective means of analyzing samples for possible toxicity. It can be used as a primary indicator of toxicity, which helps direct the process of chemical evaluation. If toxicity is detected in the assay, then further testing is needed to confirm the chemical cause of toxicity.

Bacillus licheniformis was used in this study for its ability to produce the enzyme á-glucosidase, which can be measured colorimetrically. *Bacillus licheniformis* is a Gram-positive, motile, spore-forming, facultative anaerobic rod. It produces protease, amylase, glucosidase, and other enzymes. The enzyme alpha-glucosidase is produced when alpha-amylase, a cytoplasmic enzyme, hydrolyzes starch to form maltose. Maltose, when present in extracellular fluid, induces the production of alpha-glucosidase. Alpha-glucosidase then hydrolyzes maltose to form glucose residues. During the exponential phase of growth, alpha-glucosidase is a membrane-bound enzyme. As the culture enters the stationary phase, alpha-glucosidase is released into the extracellular fluid (Thirunavukkarasu and Priest, 1983). A color indicator is added to the assay during induction of this stationary phase. Enzyme levels are detected by a yellow color that results from the breakage of alpha 1-4 linkages on the substrate chromogen, *p*-nitrophenyl-*alpha-D*-glucoside. Variations in color produced by the release of p-nitrophenol are read on a spectrophotometer.

Decreased production of alpha-glucosidase has been previously noted to occur in the presence of toxins such as metals. Therefore, samples exhibiting enzyme inhibition were analyzed for the presence of metals. Metal

concentrations were determined by the *direct air-acetylene flame method* (Clesceri et al., 1998), with an atomic absorption (AAS) spectrophotometer. This simple method only required the mixing of standards to form a calibration curve and direct analysis of samples. Previous studies indicate a correlation between enzyme production and metal concentration (Bitton et al., 1992).

MATERIALS AND METHODS

Sample collection

Drinking water samples used in this study were collected on June 13, 2000, from three homes in Lilbourn, North Lilbourn, and Howardville counties of Missouri. Water samples were collected from the Lilbourn water treatment plant, which serve these communities, before (directly from groundwater inlet) and after treatment. Samples were also obtained by request from a concerned nearby resident of New Madrid County. All samples were collected in plastic sample bottles and stored on ice until analyzed.

Cell culture preparation

To begin the assay, 50 µl of a frozen *Bacillus licheniformis* stock culture was transferred to a 5-ml test tube containing trypticase soy broth (TSB) without dextrose and incubated 24 hours at 30°C (all incubation periods were conducted at 30°C on a shaker). We transferred the overnight culture to 45 ml of fresh TSB media in a Ryan culture flask, then incubated the culture for an additional 24-hr period. The optical density (OD) of the overnight culture was then read using a spectrophotometer set at 550 nm. The culture was then diluted to an absorbance between 0.3-0.4 nm with fresh media and allowed to grow up to an absorbance between 0.7-0.8 nm. Absorbances were read and recorded every 30 min to monitor and confirm the exponential phase of the culture's development. The *Bacillus licheniformis* exponential culture was transferred to sterile centrifuge tubes and centrifuged 20 min. at 4°C at 8,000 rpms. The cell pellet was recovered and suspended in 100ml of distilled water. After vortexing the cell mixture, it was ready for immediate use in the assay.

AGB ASSAY

Exposure to toxicant

Triplicate tubes were prepared for each water sample, including triplicates for the controls, cell blanks, and sample blanks. To each tube except the cell blanks, 0.1 ml of the resuspended cells was added. To each control tube, 0.9 ml of distilled water was added. To the cell blanks and sample tubes, 0.9 ml of the collected water samples was added. After each addition (throughout the assay), all tubes were vortexed. All tubes were incubated for 60 min on a shaker at 30°C.

Enzyme induction

Maltose (0.1 ml) was added to the tubes for enzyme induction. To all tubes, 0.4 ml z-buffer (pH 7) and 0.5 ml of fresh medium were added. All tubes were incubated for 60 minutes at 30°C.

Color development

After enzyme induction, 0.1ml of 7% sodium dodeycl sulfate was added to each tube to facilitate enzyme release. A 0.4%, substrate

solution of *p*-nitrophenyl-*á-D*-glucopyranoside was added to each tube. The tubes were incubated for 60 minutes on a shaker at 30°C.

Alpha-glucosidase measurement

The reaction was stopped by adding 1 mL of cold 1-M sodium carbonate to each tube. Absorbance was measured on a spectrophotometer set at 420 nm.

Metals analysis

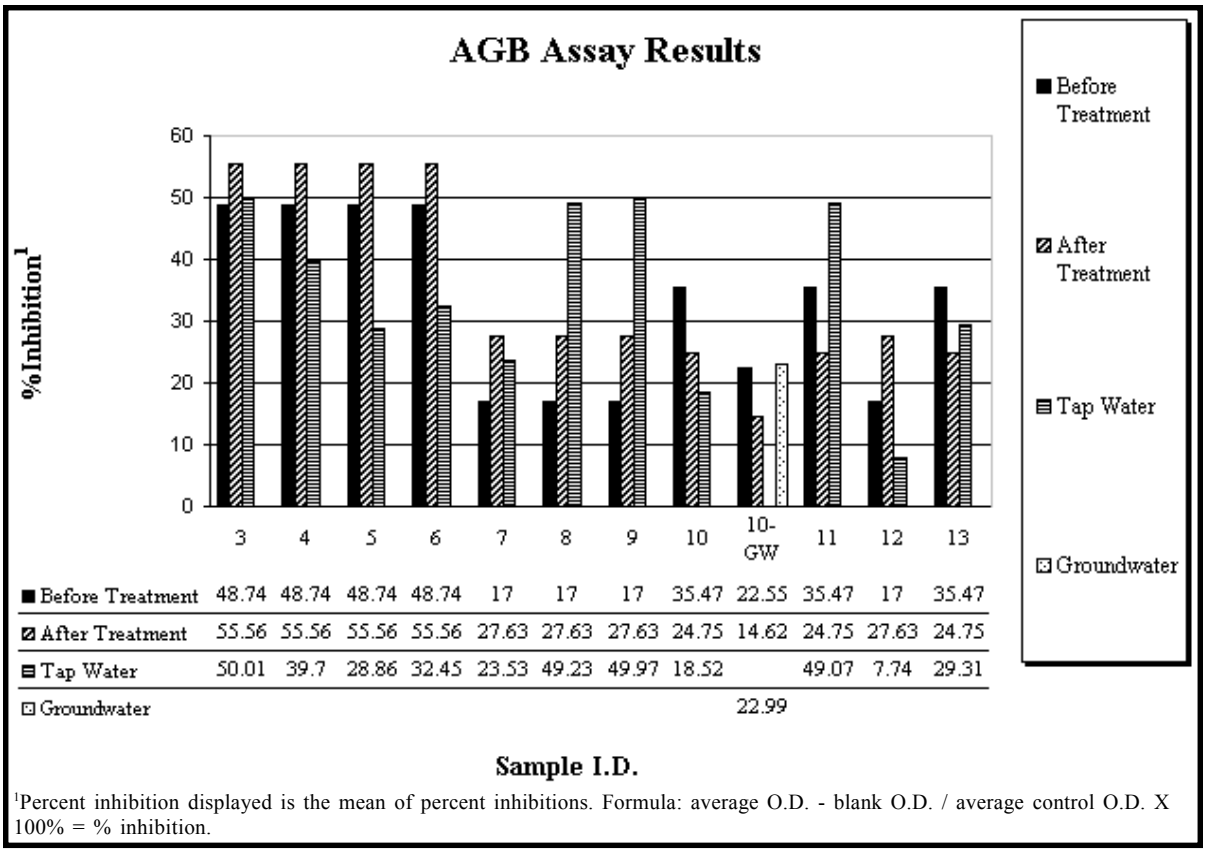
Samples were analyzed for concentrations of cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), zinc (Zn), and chromium (Cr). Calibration curve standards ranging from 0.25 mg/L (ppm) to 5.0 ppm were prepared by mixing designated amounts of purchased 1000-ppm standard solutions of each metal, with 1 ml of 1 % HNO3. Distilled water was added to the metal solutions in 100-ml volumetric flasks to fill to volume. Nitric acid was added to each flask to prevent adherence of metals to the glass. Samples were read directly from their original containers using an instrumentation laboratory - Video 22 - (AAS).

RESULTS AND DISCUSSION

AGB assay results

Results of the bioassay indicate the highest percentage inhibition in samples collected from sites 3, 8, 9, and 11, which resulted in inhibition levels above 40% [50.01%, n = 3 (in all samples); 49.23%; 49.97%; and 49.07%, respectively]. Results are recorded in Table 2. The lowest percentage inhibition levels were found in samples from sites 7, 10, 10-GW, and 12 (25.53%, 18.52%, 22.99%, and 7.74%, respectively). Higher inhibition levels may indicate toxicity by metals, while lower inhibition levels can indicate the presence of organic chemicals. Lower inhibition levels indicate the higher enzyme levels, while higher percentage inhibitions are due to lower enzyme levels.

The water treatment process at the Lilbourn water treatment plant includes sand filtering followed by chlorination. *Before treatment* (groundwater) and *after treatment* samples obtained from the pump at the Lilbourn water treatment plant were analyzed with each sample group. When comparing sample group percentage inhibitions to *before* and *after treatment* sample groups, the ***greatest differences in increases*** of inhibition occurred in samples from sites 8, 9, and 11 with differences ranging from 32.23% above *before treatment* water and 10.63% above after treatment water samples from sites 8, and 13.6% inhibition above in samples *before treatment* and 24.72% above the *after treatment* sample from site 11 (Figure 1). The ***greatest differences in decreases*** of sample percentage inhibition occurred in samples from sites 5, 6, 10, and 12. Inhibition differences ranged from 19.88% below *before treatment water* and 15.86% below *after treatment water* in the sample from site 5, to inhibitions of 9.26% below *before treatment water* and 19.89% below *after treatment* sample inhibition in the sample from site 12. Sample group 10-GW indicated a 22.99% inhibition approximately equal to that of *before treatment water* (22.55%). *After treatment water* from sample group 10-GW indicated a slightly reduced inhibition at 14.62%.



	3	4	5	6	7	8	9	10	10-GW	11	12	13
Before Treatment	48.74	48.74	48.74	48.74	17	17	17	35.47	22.55	35.47	17	35.47
After Treatment	55.56	55.56	55.56	55.56	27.63	27.63	27.63	24.75	14.62	24.75	27.63	24.75
Tap Water	50.01	39.7	28.86	32.45	23.53	49.23	49.97	18.52		49.07	7.74	29.31
Groundwater									22.99			

[1]Percent inhibition displayed is the mean of percent inhibitions. Formula: average O.D. - blank O.D. / average control O.D. X 100% = % inhibition.

Figure 1. Percent inhibition results of the AGB assay for water samples collected from the Lilbourn water treatment facility and homes in southeastern Missouri.

Results of our AGB bioassay suggest that water being served to Lilbourn, Howardville, New Madrid, and North Lilbourn counties of southeast Missouri may contain organic chemicals and variable metal concentrations (Figure 1). We did not test further for organic chemicals (pesticides) at this time. However, we do plan to analyze tap water and groundwater collected from these sites for pesticide concentrations in future studies. In this study*, we analyzed the samples for metal concentrations of cadmium, copper, lead, zinc, and chromium* (Table 1). Results of the metal analysis reveal concentrations of cadmium, chromium, and lead, which slightly exceeded EPA's national MCLG's for these metals (Table 2).

Cadmium is a highly toxic element. In the human body its absorption is enhanced by dietary deficiencies of calcium and iron. A population study, which included 2,327 participants of four districts in Belgium, concluded that cadmium intake leads to cardiovascular diseases as effect on tubular function of the kidneys (Staessen and Lauwerys, 1993). Some possible contaminant sources of Cd in drinking water are corrosion of galvanized pipes, erosion of natural deposits, discharge from metal refineries, and runoff from waste batteries and paints (EPA, 2001). Possible health effects resulting from ingestion of excess Cr in drinking water over prolonged periods of time could result in allergic dermatitis and cancer (Rowbothan and Shuker, 2000). Possible sources of drinking water contamination from Cr include discharge from steel and pulp mills,

and erosion of natural deposits (EPA, 2001). Pb exposure is most prevalent today. A study on malnutrition of a minority population in China revealed that lead poisoning causes delays in physical or mental development in children, and kidney dysfunctions and high blood pressure in adults (Srianujata, 1998). Some possible ways that lead enters water sources are corrosion of household plumbing systems and erosion of natural deposits (EPA 2001).

Metal analysis results

Metal concentrations detected in water samples collected for this study are reported in Table 1. EPA national maximum contaminant level goals (MCLG's) for these metals are Cd = 0.005 mg/L (ppm), Cu =1.3 ppm, Pb = 0.0 ppm, Zn =5.0 ppm, and Cr = 0.100 ppm (Meranger et al., 1981).

Cd levels were found to be below MCLG's in samples from sites 5, 8, 10, and 11. Cu and Zn in all samples were observed and also found to be below MCLG's. Samples with metal concentrations found to exceed EPA's national MCLG's contained Cd, Cr, and Pb (Table 2). Cd concentrations were greater than MCLG's (> 0.005 ppm) in before and after treatment water obtained from the Lilbourn water treatment plant, and from homes at sites

Table 1. Concentrations of Cd, Cu, Ni, Pb, Zn, and Cr in before and after treatment samples from the Lilbourn water treatment plant, and tap water and groundwater samples obtained from homes in southeastern Missouri counties, as determined by atomic absorption spectrometry.

County	Water Type[1]	Sample	Cd, mg/L	Cu, mg/L	Pb, mg/L	Zn, mg/L	Cr, mg/L
Lilbourn	BT	1	0.008±0.019	0.015±0.009	0.014±0.108	-0.008_0.010	0.461_0.068
Water Treatment Plant	AT	2	0.009±0.022	0.017±0.008	0.047±0.269	-0.020_0.009	0.378_0.047
Howardville	T	10	0.004±0.012	0.008±0.004	0.174±0.206	0.080±0.012	0.045±0.100
	T	8	0.005±0.006	-0.007±0.011	0.137±0.120	0.003±0.015	0.046±0.032
	T	9	0.024±0.020	0.019±0.010	0.102±0.106	0.092±0.009	0.036±0.056
	GW	10	0.014±0.009	-0.003±0.010	-0.128±0.150	0.100±0.007	0.111±0.032
Lilbourn	T	13	0.023±0.026	0.254±0.008	-0.019±0.029	0.113±0.012	0.444±0.037
	T	7	0.009±0.021	0.036±0.008	-0.053±0.136	-0.006±0.010	0.365±0.042
	T	6	0.022±0.030	0.042±0.013	0.130±0.191	-0.006±0.007	0.418±0.049
New Madrid	T	11	0.023±0.012	0.022±0.034	0.177±0.082	0.026±0.003	0.125±0.029
North Lilbourn	T	4	0.015±0.001	-0.006±0.020	0.133±0.183	0.024±0.010	0.055±0.081
	T	3	0.027±0.021	0.017±0.014	0.269±0.140	0.009±0.007	0.085±0.091
	T	5	0.002±0.003	0.014±0.013	-0.015±0.228	0.010±0.010	1.433±0.043

[1]BT=water before treatment; AT=water after treatment; T=tap water; GW=groundwater. Site I.D.=sample collected at this site.
[2]Concentrations are listed in mg/L (ppm). Concentrations listed are the means of 10 successive readings. Concentration and standard deviation were calculated by the AAS.

4, 6, 7, 9, 11, and 13. Toxic Cd levels detected exceeded MCLG's by 0.003 to 0.022 ppm.

Concentrations of Cr were also found to exceed EPA's national standards with concentrations greater than (> 0.100 ppm) in before and after treatment water, as well as in tap water obtained from homes at sites 5, 6, 7, 11, and 13. Chromium levels found at these sites exceeded national goals by 0.011 to 0.361 ppm. Concentrations above .500 ppm were beyond detection limits.

Results from the Pb analysis also indicated *high* concentrations. Pb concentrations ranged from 0.014 to 0.047 ppm in before and after treatment water. Samples from sites 5, 7, 10, and 13 were below detection levels. All other samples were detected to contain Pb levels from 0.102 to 0.269 ppm. The EPA national MCLG for Pb concentration in drinking water is zero.

CONCLUSIONS

The goal of this study was to gather information about the quality of water being distributed to selected southeastern Missouri communities. Since the communities studied are farm communities, our results are not surprising. The presence of metals such as lead in some samples are a major concern because of its effects on the health of children. The data

Table 2. Percent inhibition (all samples) and metal concentrations in samples that exceeded the national MCLG set by EPA for before and after treatment samples from the Lilbourn water treatment plant, tap water, and groundwater samples obtained from homes in southeastern Missouri counties.

County	Water Type[1]	Sample	AGB Assay, % Inhibition	Cd, mg/L[2]	Cr, mg/L[2]	Pb, mg/L[2]
Lilbourn	BT	1	48.74	0.008	0.461	0.014
Water Treatment Plant	AT	2	55.56	0.009	0.378	0.047
Howardville	T	10	18.52	--	--	0.174
	T	8	49.23	--	--	0.137
	T	9	49.97	0.024	--	0.102
	GW	10	22.99	0.014	0.111	--
Lilbourn	T	13	29.31	0.023	0.444	--
	T	7	23.53	0.009	0.365	--
	T	6	32.45	0.022	0.418	0.130
New Madrid	T	11	49.07	0.023	0.125	0.177
North Lilbourn	T	4	39.70	0.015	--	0.133
	T	3	50.01	0.027	--	0.269
	T	5	28.86	--	--	--

[1]BT= water before treatment (raw); A= water after treatment (treated); T= tap water (distributed water): GW= groundwater.
[2]Metal concentrations are listed in mg/L (ppm). Concentrations listed are the means of 10 successive readings. Concentration and standard deviation were calculated by the AAS.
MCLG: Cd 0.005 mg/L, Cu 1.3 mg/L, Pb 0.000 mg/L, Zn mg/L, Cr mg/L.

collected in this study and from future studies will be disseminated to the community leaders so that strategies can be developed to address drinking water contamination. Strategies should include improved monitoring and treatment procedures to remove metals contaminants.

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REFERENCES

Bitton, G., M. Campbell, and B. Koopman. 1992. MetPad: A Bioassay Kit for the Specific Determination of Heavy Metal Toxicity in Sediments from Hazardous Waste Sites. Environ Toxicol and Water Qual. 7: 323-328.

Clesceri, L. S., A. E. Greenberg, and A.D. Eaton. 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed.

EPA Electronic Publication (2001). National Primary Drinking Water Standards. www.epa.gov/safewater/mcl.html

Lauwerys, P. Lijnen, and H. Roels. 1992. *Effects of Cadmium Exposure on the Cardiovascular System and on Calcium Metabolism: Results of a Cross-Sectional Population Study*. IARC Sci Publ. 1992. 118: 263-269.

Méranger, J. C., K. S. Subramanian, and C. Chalifoux. 1981. Metals and Other Elements: Survey for Cadmium, Cobalt, Chromium, Copper, Nickel, Lead, Zinc, Calcium, and Magnesium in Canadian Drinking Water Supplies. J. Assoc. Off. Anal. Chem. 64:1: 44-53.

Rowbotham, A. L., L. S. Levy, and L. K. Shuker. 2000. Chromium in the Environment: An Evaluation of Exposure of the UK General Population and Possible Adverse Health Effects. J Toxicol Environ Health B Crit Rev. 3:3: 145-178.

Staessen, J., A. Bernard, J. P. Buchet, F. Claeys, L. Dekempeneer, G. Ducoffre, R. Fagard, and R. Lauwerys. 1993. Health Effects of Environmental Exposure to Cadmium in a Population Study. J Hum Hypertens. 195-199.

Srianujata, S., 1998. Lead—The Toxic Metal to Stay with Humans. J Toxicol Sci. 2: 237-240.

Thirunavukkarasu, M., and F. G. Priest. 1983. Synthesis of á- Amylase and Alpha-Glucosidase by Membrane-Bound Ribosomes from *Bacillus licheniformis*. Biochem Biophys Res Commun. 114:677-683.