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Rapid Respirometric Toxicity Test: Sensitivity to Metals

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Due to increased industrialization, as well as the increased demand for chemicals, both developed and developing nations face increasing ecological and toxicological problems from the release of toxic contaminants to the environment. Research efforts are being directed at the development of short-term bioassay tests, in an attempt to alert dischargers as well as monitoring agencies to potentially toxic conditions (Dutka et al. 1983). Bacteria and yeast have several attributes which make them attractive as test organisms for the rapid screening of chemical pollution in natural waters. They have relatively short life cycles and, therefore, respond rapidly to environmental change. They are easily handled and inexpensively maintained. Their rate of multiplication is such that a large number of homogeneous individuals are avalaible for use in toxicity test procedures (Bitton 1983).

One current approach for assessing cytotoxicity is to monitor respiratory activity, a sensitive, nonspecific subcellular target site (Haubenstricker et al. 1990). Microbial dehydrogenase activity can be used to evaluate microbial viability because in microbial respiration, the dehydrogenases participate in the transport of electrons from substrate to final electron acceptors in an electron transport system (ETS). Tetrazolium salts act as artificial electron acceptors along ETS, becoming reduced to form insoluble formazans; therefore, microbial activity can be assessed using 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) reduction to the photometrically measured end product, INT-formazan (Dutton et al. 1986).

The purpose of the present study was to develop a simple, rapid and practical toxicity test, based on monitoring changes in respiratory activity of *Saccharomyces cerevisae* and *Pseudomonas fluorescens*, and to avoid the tedious counts necessary for baker's yeast assay while improving upon the sensitivity of the test, for potential inclusion in a battery of short-term microbial toxicity screening assays. The sensitivity and suitability of the test was evaluated using several heavy metal salt solutions and heavy-metal polluted wastewater, and comparing data with those obtained using the baker's yeast assay.

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MATERIALS AND METHODS

The toxicity of the following heavy metals was investigated: Cr^{6+} (K_2CrO_4), Cu^{2+} ($CuCl_2.2H_2O$), Cd^{2+} ($CdCl_2$), Hg^{2+} ($HgCl_2$), Ni^{2+} ($NiCl_2$) and Zn^{2+} ($ZnCl_2$). Deionized water and wastewater were used for the preparation of chemical solutions and for control tests. Stocks solutions were stored at 4°C. A commercial brand of dried baker's yeast and a *P. fluorescens* strain, obtained from the Spanish Type Culture Collection (CECT nº 385, ATCC nº 13525), were used as test organisms. Lyophilized yeast and frozen bacterial cultures held at -30°C and -80°C, respectively, were employed.

Baker's yeast assay (Bitton et al. 1984) was carried out by preparing a 1% (v/v) suspension of yeast in sterilized saline solution (0.85% NaCl) as the suspending fluid. The yeast suspension was stirred for 15 min to break up yeast floc. Toxicant (0.2 mL) was added to 0.8 mL of yeast suspension and incubated for 30 min at 30°C with shaking. To this, 0.1 mL of INT solution (0.2%) and 0.1 mL of 10% solution of yeast extract were added and the mixture incubated in the dark at 30°C for 1 hr with shaking. The reaction was stopped with 0.1 mL of 37% formaldehyde. The proportion of respiring cells was determined as follows: one or two loopfuls of the yeast suspension was spread on a glass slide, air dried, counterstained with 0.025% malachite green and blotted after 1 min; 500 cells were examined with bright field microscopy (100x) and the number of respiring cells (green cells with red INT-formazan crystals) and non respiring cells (green cells) were determined. Results were expressed as percent inhibition compared with negative controls. EC20 and EC50 values (concentrations exhibiting 20 and 50% reduction in the percentage of respiring cells, respectively) were calculated using regression analysis.

Spectrophotometric assays used in this work were a modification of the method of Dutton (1983) using *P. fluorescens*. The bacteria were incubated overnight (10hr) with slight shaking in nutrient broth until the optical density of culture at 650 nm was 0.3-0.4. An aliquot (0.8 mL) of the bacterial culture was incubated with the toxicant (0.2 mL) for 1 hr at 30°C with shaking. Then 0.1 mL of 0.2% INT was added and the samples were incubated in the dark at 30°C with shaking for 1 hr. The sample was fixed with formalin, centrifuged at 2000 g for 20 min and the supernatant was decanted. The pellet was extracted with 2 mL of DMSO and vortexed for 15-30 sec to disrupt the pellets. The extract was centrifuged at 2000 g for 20 min and the optical density of the supernatant was read at 460 nm. The degree of inhibition was evaluated by expressing absorbance values as percentage of the negative control. The protocol for the spectrophotometric assay using *S. cerevisiae* was similar to that of the baker's yeast assay until the addition of formalin. After this point the procedure was similar to the spectrophotometric assay using *P. fluorescens,* except for a shorter centrifugation (10 min instead 20). EC20 and EC50 values were calculated using regression analysis.

RESULTS AND DISCUSSION

Table 1 shows the effect of some heavy metals used at different doses on the respiratory activity of *S. cerevisiae* and *P. fluorescens* using both microscopic and spectrophotometric assays. A significant correlation was observed between toxicant concentration and respiratory activity inhibition for all heavy metals tested with the respirometric assays. In general, the respiratory activity response curve was linear. However, the respiratory activity response curve for Cr^{6+} was exponential (Fig. 1) and for Ni^{2+} was non-linear only using the microscopic assay, where concentrations of Ni^{2+} ≤2000 mg/L had no effect, and concentrations >2500 mg/L exhibited total inhibition.

Table 1. Respiratory activity responses and correlation coeficients of three respirometric assays to different heavy metals.

Toxicant	Baker's yeast assay		Spectrophotometric assays			
			S. cerevisiae		*P. fluorescens*	
	Response	r	Response	r	Response	r
Cr	E	0.92**	E	0.99*	E	0.99*
Cu	E	0.95**	L	0.99*	L	0.99*
Cd	L	0.99*	L	0.99*	L	0.98*
Hg	L	0.96**	L	0.98*	L	0.99*
Ni	N-L	-	L	0.98*	L	0.99*
Zn	L	0.99**	L	0.99*	L	0.98*

E: Exponential. L: Linear. N-L: Non-linear. *: p≤0.001 **: p≤0.01

Table 2 shows a comparison of the relative sensitivity of three respirometric assays to several heavy metals. Spectrophotometric assays were more sensitive than microscopic assay. A comparison of EC20 and EC50 values show that spectrophotometric yeast assay was more sensitive than the microscopic assay to Cr^{6+}, Hg^{2+}, Ni^{2+} and Zn^{2+}, and equally sensitive to Cu^{2+} and Cd^{2+}. However, *P. fluorescens* respirometric assay showed a higher sensitivity than the spectrophotometric yeast assay to Hg^{2+}, Cd^{2+} and Ni^{2+} and Zn^{2+} and was less sensitive to Cr^{6+} and Cu^{2+}. Bitton et al. (1984) obtained lower EC50 values for some of the heavy metals studied using the microscopic yeast assay.

Spectrophotometric yeast assay was more sensitive than Microtox assay based on bacterial bioluminescence and *Spirillum volutans* motility test to Cr^{6+} and *P. fluorescens* assay was more sensitive to Cd^{2+} than Microtox. Spectrophotometric assays were less sensitive to the other heavy metal tested than Microtox and *S. volutans* assays which are more expensive and sophisticated than respirometric assays (Dutka and Kwan 1981; Goatcher et al. 1984; Qureshi et al. 1984; Greene et al. 1985; Paran et al. 1990).

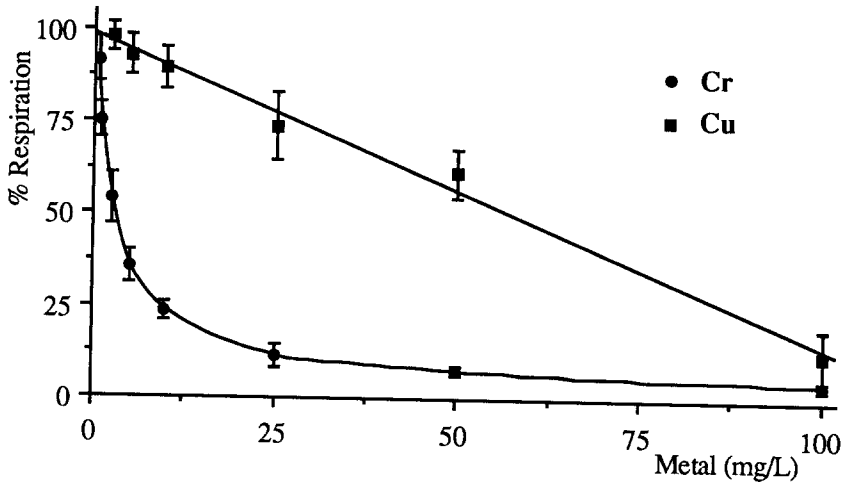

Figure 1. Effects of chromium and copper on the respiratory activity of *S. cerevisiae* and *P. fluorescens*, respectively, using spectrophotometric assays.

Table 2. Sensitivity comparison of respirometric assays to heavy metals (mg/L).

Toxicant	Baker's yeast assay		Spectrophotometric assays			
			S. cerevisiae		*P. fluorescens*	
	EC20	EC50	EC20	EC50	EC20	EC50
Cr	4.2	8.3	0.8	3.3	3.8	22.0
Cu	13.6	27.2	11.3	29.3	20.1	56.1
Cd	151	292	118	295	10.1	25.9
Hg	66.6	126	30.0	75.0	1.2	6.6
Ni	>2000*	>2000*	732	1515	65.3	188
Zn	111	264	30.3	78.3	19.5	35.7

*<2500.

Table 3 shows a comparison of the results of spectrophotometric assays on heavy-metal polluted wastewater with the EC20 and EC50 values obtained earlier with heavy metal salt solutions (Table 2). The spectrophotometric assay with *P. fluorescens* was markedly less sensitive when heavy-metal polluted wastewater was tested. The spectrophotometric yeast assay was equally sensitive to Cr^{6+}, Cu^{2+}, Cd^{2+} and Hg^{2+}, and less sensitive to Ni^{2+} and Zn^{2+}. However, the *P. fluorescens* respiratory activity assay exhibited greater sensitivity for 4 of the 6 metals tested. The spectrophotometric assay with *P. fluorescens* was more sensitive to Hg^{2+}, Ni^{2+} and Zn^{2+} and less sensitive than yeast assay to Cr^{6+} and Cu^{2+}, when metal-polluted wastewater was studied.

Table 3. Toxicity assessment of effluents with heavy metal addition using spectrophotometric assays (mg/L).

Toxicant	*S. cerevisiae*				*P. fluorescens*			
	EC20		EC50		EC20		EC50	
	S.	W.	S.	W.	S.	W.	S.	W.
Cr	0.8	0.4	3.3	3.1	3.8	8.4	22.0	54.9
Cu	11.3	11.7	29.3	39.8	20.1	40.6	56.1	72.2
Cd	118	82.7	295	279	10.1	>100	25.9	>100
Hg	30.0	47.6	75	110	1.2	6.7	6.6	13.9
Ni	732	1322	1515	2349	65.3	182	188	320
Zn	30.3	36.7	78.3	208	19.5	72.8	35.7	110

S: Heavy metal solution. W: Heavy-metal polluted wastewater.

A simple, rapid and reproducible assay based on respiratory activity inhibition of *S. cerevisiae* and *P. fluorescens* was developed and evaluated for toxicity screening and assessment. It differs from other respiratory related microbial tests in that it uses a spectrophotometric determination. The proposed assay represents improvements in terms of relatively low cost, greater sensitivity and short duration (3h).

Data from the heavy metals testing indicated that the spectrophotometric assay is potentially useful for the detection of chemical toxicity. The spectrophotometric yeast assay appeared to be more sensitive than microscopic assay, but was generally less sensitive than the *P. fluorescens* respiratory activity inhibition test. Yeast assay, however, showed greater sensitivity than *P. fluorescens* to Cr^{6+} and Cu^{2+}. The respiratory activity inhibition of these microorganisms by an influent wastewater stream could be monitored more easily at regular time intervals, allowing protective measures to be initiated before treatment processes are interrupted by toxic inputs.

Coleman and Qureshi (1985) and Paran et al. (1990) have suggested the use and application of a battery of test approaches for biological testing and toxicity screening, particulary because there is no universal test or system that can detect all toxicants to adequately assess the wide range of ecotoxicological effects.

The observed different sensitivity patterns of *S. cerevisiae* and *P. fluorescens* assays further support the concept of using a battery of short-term screening tests for chemical and environmental toxicity assessment. The results of this study also indicate that the respirometric assay with *S. cerevisiae* and *P. fluorescens* could be an important component of such a battery.

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